Universidade de Lisboa

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Distinct strategies to overcome severe forms of PKU: The p.G46S as a model to identify small molecules modulators of protein aggregation and evaluation of an enzyme replacement approach using a nanoparticulate system

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Dissertação orientada pela Professora Doutora Ana Paula Costa dos Santos Peralta Leandro e, coorientada pelo Doutor João Paulo Travassos Leandro

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Abstract

This work explores distinct strategies to overcome phenylketonuria (PKU; OMIM 261600), the most common autosomal recessive disorder of amino acid metabolism caused by a deficiency of the hepatic human phenylalanine hydroxylase enzyme (hPAH; EC 1.14.16.1) and for which the available therapies rely mainly in a dietetic restriction. PKU is considered a conformational disease, with loss-of-function, where the normal balance between folding and degradation machineries (proteostasis) is displaced towards the accelerated degradation of the misfolded hPAH variants due to their decreased stability and high tendency to aggregate. Therefore, small molecules modulating either the conformation (pharmacological chaperones, PC) or the interaction of misfolded proteins with the cellular pathways involved in protein homeostasis (proteostasis regulators; PR) might enhance the concentration and/or location of the target proteins thus contributing to alleviate disease pathogenesis. From the more than 600 different disease-causing mutations identified to date, the hPAH p.G46S is an excellent model of aggregation-prone variants as it promotes self-association and fibril formation *in vitro*. Alternatively, enzyme reposition therapy (ERT) would be an universal therapeutic approach as it could rescue the full spectrum of PKU phenotypes. As such, this project aimed to: (i) hint a new class of PC, from an in-house compound library, inhibiting hPAH aggregation and using p.G46S as the study model; (ii) optimize the experimental conditions for further studies to validate PR involved in p.G46S degradation and; (iii) evaluate a potential drug delivery system, previously developed by the research group aiming to develop a ERT for PKU.

By performing *in vitro* aggregation studies on recombinantly produced p.G46S and immunocytochemistry assays on transfected eukaryotic cells, two 3-hydroxyquinolin-2(1H)-one derivatives (C6 and C18) were identified as aggregation inhibitors. These are promising compounds to be used as scaffolds for further structure refinement. Additionally, co-transfection and co-immunoprecipitation experimental conditions were established in order to study the interactions of molecular chaperones and p.G46S, a fundamental step to further identify PR. Our assays, regarding the protective effect of chitosan-nanoparticles showed that in fact the nanoencapsulated hPAHwt presented a higher activity (after 4 h incubation in culture medium, at 37°C) than the naked protein (in the same experimental conditions).

Overall, the obtained results will allow to continue to pursuit novel therapeutic strategies to PKU, which in case of the adaptation of cellular proteostasis approach, might be transversal to others conformational disorders.

Key words: Inborn errors of metabolism, Phenylketonuria, Pharmacological Chaperones, Proteostasis Regulators, Chitosan Nanoparticles

Resumo

Este trabalho explora diferentes estratégias para tratamento da fenilcetonúria (PKU; OMIM 261600), o erro hereditário do metabolismo dos aminoácidos mais comum, o qual é causado pela deficiência da enzima fenilalanina hidroxilase (hPAH; EC 1.14.16.1) e onde a restrição dietética continua a ser a principal abordagem terapêutica. A PKU é considerada uma doença conformacional, por *loss-of-function* da hPAH, em que o balanço normal entre o *folding* e a maquinaria de degradação (proteostase) se encontra deslocado no sentido de acelerar a degradação de proteínas *misfolded* devido à sua instabilidade e tendência para agregação. Assim, pequenas moléculas que modulem a conformação (chaperones farmacologócios, PC) ou a interação de proteínas *misfolded* com as vias celulares envolvidas na homeostase (reguladores da proteostase; PR) poderão modular a quantidade e/ou localização de proteínas alvo e desta forma, contribuírem para melhorar a patogénese da doença. Das mais de 600 mutações diferentes, identificadas até à data como causadoras de PKU, a hPAH p.G46S é um excelente modelo de variantes associadas a agregação proteica uma vez que, *in vitro*, promove auto-associação e formação de fibrilas. Alternativamente, a terapia enzimática de reposição (ERT) seria uma abordagem terapêutica universal, pois poderia atuar sobre qualquer fenótipo PKU. Deste modo, este projeto teve como objetivo: (i) desenvolver uma nova classe de PC, a partir de uma biblioteca de compostos sintetizados *in-house*, com capacidade para inibir a agregação da hPAH, usando a variante p.G46S como modelo de estudo; (ii) otimizar as condições experimentais para identificar as vias de proteostase envolvidos na degradação da p.G46S de modo a identificar PR e; (iii) avaliar um potencial sistema de entrega de fármacos, previamente desenvolvido pelo grupo de investigação, que permita alcançar uma abordagem terapêutica para a PKU através de ERT. A p.G46S foi produzida em *E. coli* e os ensaios de agregação permitiram identificar dois derivados 3-hydroxyquinolin-2(1H)-one (C6 e C18) inibidores de agregação. Estes resultados foram corroborados em ensaios *in cellulo*, onde os mesmos compostos demonstraram ser promissores para serem utilizados como *scaffold* para refinamento da estrutura molecular. Além disso, foram otimizadas as condições experimentais de co-transfeção e co-imunoprecipitação que permitirão estudar as interações entre chaperones moleculares e a p.G46S, um passo fundamental para a futura identificação de PR. Relativamente ao efeito protetor das nanoparticulas de quitosano, a proteína hPAHwt nanoencapsulada apresentou maior atividade (após 4 h de incubação em meio de cultura, a 37 ° C) do que a proteína livre (nas mesmas condições experimentais).

Em suma, os resultados obtidos com este trabalho permitirão continuar em busca de estratégias terapêuticas para a PKU. De realçar que os estudos que envolvam a proteostase celular, poderão ser transversais a outras doenças conformacionais.

Palavras chave: Erros hereditários do metabolismo, Fenilcetonúria, Chaperones Farmacológicos, Reguladores da Proteostase, Nanopartículas de quitosano

Abbreviations

ACT – Aspartase kinase, Chorismate mutase and TyrA
AAAHs – Aromatic Amino Acid Hydroxilases
ASO – Antisense Oligonucleotide therapy
BCA – Bicinchoninic acid
BH4 – 6(R)-L-erythro-5,6,7,8-tetrahydrobiopterin
BSA – bovine serum albumin
CS – Chitosan
DDS – Drug Delivery System
DLS – dynamic light scattering
DMSO – dimethyl sulfoxide
DTT – dithiothreitol
ERT – Enzyme Replacement Therapy
EURODIS – European Organization for Rare Diseases
FXa – factor Xa
GMP - Glycomacropeptide
HPA – Hyperphenylalalinemia
hPAH – human phenylalanine hydroxilase
hPAHwt - human phenylalanine hydroxilase wild type
hPAH G46S – human phenylalanine hydroxilase mutant G46S
HPLC – high performance liquid chromatography
Hsp – Heat shock protein
IEM – Inborn Error of Metabolism
IMAC – Immobilized Metal Affinity Chromatography
IMD – Inherited Metabolic Disorders
IPTG – isopropyl-β-D-thiogalactoside
LNAs – Locked Nuclei Acids
LNAAs – Large Neutral Amino Acids
L-Phe – L-phenylalanine
L-Trp – L-tryptophan
L-Tyr – L-tyrosine
MBP – maltose binding protein
Min – Minute(s)
MM – molecular mass
NP – Nanoparticle
ON – Overnight
PAL – Phenylalanine Ammonia Lyase
PC – pharmacological chaperone

PEG – Polyethylene glycol

PEI - Polyethylenimine

PKU – Phenylketonuria

PN – Proteostasis Network

PR – proteostasis regulator

PTC – Premature Termination Codon

SDS-PAGE – sulphate polyacrilamide gel electrophoresis

SEC – size exclusion chromatography

tlag – lag time

TPP - Tripolyphosphate

UPR – Unfolded Protein Response

λ – wavelenght

CHAPTER I • **INTRODUCTION**

1. NEW APPROACHES TO THE TREATMENT OF INBORN ERRORS OF METABOLISM

The term “inborn error of metabolism” (IEM) was firstly presented by Sir Archibald E. Garrod, in 1908 during his studies on alkaptonuria (1) . Since then, the number of diseases identified as IEM has increased, due to the development of new diagnostic techniques for the various biochemical phenotypes, and, since then, more than five hundred different IEM have been described (2).

Inborn errors of metabolism, or inherited metabolic disorders (IMD), are monogenic disorders and classically they result from the lack or impaired activity of a specific enzyme or a transport protein. The consequences of the protein deficiency include: (i) accumulation of the enzyme substrate, usually present in small amounts; (ii) the deficiency of critical intermediary products; (iii) the deficiency of specific final products; (iv) the toxic excess of products of alternative metabolic pathways (3). The differences in the phenotypic manifestations observed depend on the severity of the underlying gene mutation, the type and function of the affected protein, post translation mechanisms, cellular processes, genes in other *loci* that may also be affected and environmental factors. For all these factors, patients with IMD present a complex assembly of symptoms and some IEM are thus considered multifactorial (2).

IMDs are classified as rare disorders and according to the European Organization for Rare Disease (EURODIS), a disease is considered rare when it affects less than 1 in every 2,000 inhabitants. About 6,000 to 8,000 rare disorders have been described and although the individual frequencies are low, collectively IMD affect around 6 to 8% of the EU population (4).

As stated above, IMDs are inherited in a monogenic or Mendelian form, since only one gene plays a predominant role in the determination of disease. The majority of IMDs (67%) are of autosomal recessive inheritance, 21% are of autosomal dominant inheritance, while 6% are X-linked and another 6% are related with defects in mitochondrial genome. In general, a high heterogeneity is observed and the kind and intensity of clinical manifestations are related to the type of mutations detected (5).

As monogenic disorders, IMDs gene mutations can be classified into four major groups: missense, nonsense, splicing and frameshift. Missense mutations are typically single nucleotide changes that either alter the amino acid in the translated protein or do not alter the amino acid (silent mutation). Nonsense mutations are point mutations in a sequence that create a premature stop codon (UAA, UAG or UGA) in the coding region of the mRNA, resulting in premature translation termination and, usually, a non-functional or rapidly degraded protein is expressed. Splicing mutations result in disruption of critical sequences for splicing and abolishment of the usual splice sites, or creation of aberrant or cryptic splice sites, which in turn resulting in aberrant proteins. Finally, frameshift mutations are commonly caused by deletion or insertion of a number of nucleotides that alter the reading frame for any

subsequent downstream codons (6).

According to the pathophysiology, IMDs can be divided into three main categories; (i) disorders that give rise to intoxication, (ii) disorders involving energy metabolism and (iii) disorders involving complex molecules (7). The first group comprises inborn errors of intermediary metabolism since usually they are characterized by an acute or progressive intoxication due to a metabolic block and accumulation of toxic compounds. This group also includes inborn errors of amino acid catabolism, such as phenylketonuria (7).

In terms of therapy, the main goal in a IMD is to re-establish the metabolic balance and for that, many strategies can be applied either isolated or in combination. The first approach is to either decrease the accumulating substrate with diet restriction or to inhibit the activity of enzymes involved in preceding steps of the metabolic pathway. Other common strategies are the fast elimination of the toxic products from the body, an increase of the residual enzymatic activity (e.g. by cofactor administration) and the supplementation with the reaction end product in shortage (8).

Considering IMD medical care, the treatment represents a significant challenge to the public system, even with the scientific achievements of the last years. Until now the majority of the available therapies is not definitive and only ameliorates the patient's symptoms. For these reasons, several novel pharmacological treatments are arising and being largely investigated. They can be divided into two main groups: mutation specific therapies and non-mutation specific therapies.

1.1. MUTATION SPECIFIC THERAPY

The existence of a common type of mutations prompted the hypothesis of common type-specific molecular pathogenesis, in other words, nonsense, splicing and frameshift mutations usually lead to loss of or unstable protein whereas missense and in-frame insertion or deletion mutations produce nonfunctional or partially functional proteins. The comprehension of the underlying molecular mechanism associated with each mutation type led to the development of approaches based in the mutations nature, so that a therapeutic strategy developed against a certain type of mutation will be effective against similar mutations, regardless the gene (6,9).

1.1.1. PREMATURE TERMINATION CODON READ-THROUGH THERAPY

Approximately 1800 inherited human diseases are caused by nonsense mutations thus leading to the presence of a premature termination codon (PTC) in the transcript (10). Suppressing, by reading through the resulting PTC with compounds, allowing translation to continue to the true end of the transcript, is a promising approach for correcting this type of mutations (Figure I.1). The read-through compounds reduce ribosome termination at the PTC,

resulting in the insertion of a random amino acid and the translation of the remainder of the correct full length protein (11). PTCs that occur more than 50 nucleotides upstream of the final exon-intron junction generally induce transcript degradation through the nonsense-mediated mRNA decay surveillance pathway (12), which make any nonsense mutation that does not trigger significant nonsense-mediated mRNA decay a good candidate for this approach.

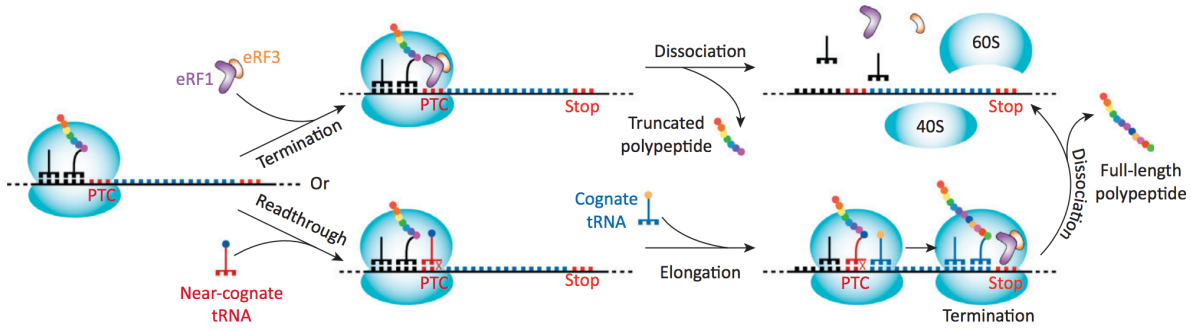


FIGURE I.1. Representation of the termination and readthrough processes. Termination is a process that occurs when a ribosome encounters a PTC and the release factors eRF1 and eRF3 enter the ribosomal A site to promote translation termination and ribosomal subunits dissociation. Readthrough is an alternative process where a near-cognate tRNA forms a less-than-optimal interaction with a codon decoding the PTC, leading the ribosome to carry on translation until reaching the next stop codon. Adapted from (13).

One of the PTC read-through compounds are aminoglycoside antibiotics. They bind to the decoding site of the 16S ribosomal RNA, inducing a local conformational change that allows translation through what would otherwise be read as a PTC (14). Paromomycin, geneticin (G418) and gentamicin are aminoglycosides that have demonstrated to partially restore the full-length protein of nonsense mutations in mammalian cells (paromomycin and G418) (14) and in clinical trials for the treatment of cystic fibrosis, Duchenne muscular dystrophy and hemophilia (gentamicin) (15,16). However, for an effective read-through a high concentration of aminoglycosides is needed which is often toxic to cells and consequently restricts their uses in humans. Furthermore, most antibiotics do not cross the blood-brain barrier efficiently and would be of limited use for treating central nervous system diseases. To reduce aminoglycosides' cell toxicity while retaining the read-through activity, some attempts have been made in redesign their structure (17).

The non-aminoglycoside antibiotic PTC124 is a nonsense-suppression read-through compound recently identified, that was systemic delivered into mouse models for Duchenne muscular dystrophy and cystic fibrosis showing restoration of protein and function *in vivo* without any obvious toxicity (18). PTC124 has demonstrated specific read-through activity to the PTC rather than to the true termination codons, promoting selective and specific read-through of disease-causing premature stop codons (18). Du L et al., have identified more than

50 non-aminoglycoside chemicals with read-through activity which may contribute to a rapid expansion of the discovery aspect of read-through chemicals (19).

1.1.2. ANTISENSE OLIGONUCLEOTIDE THERAPY

Antisense oligonucleotide therapy is based on an antisense oligonucleotide (ASO), which are single-stranded deoxyribonucleotides (typically 20 bp in length) that are complementary to the target mRNA (20). Hybridization of ASO to the target mRNA via Watson-Crick base pairing can result in specific inhibition of gene expression by various mechanisms, depending on the chemical make-up of the ASO and location of hybridization, resulting in reduced levels of translation of the target transcript. The ASO is not only a useful tool for studies of loss-of-gene function and target validation, but also highly valuable as a strategy to treat genetic diseases in which decreasing the levels of a mutant protein would favourably alter the phenotype (9). ASO induced protein knockdown is usually achieved by induction of RNase H endonuclease activity that cleaves the RNA phosphodiester bonds of the RNA–DNA heteroduplex, leading to the degradation of target mRNA while leaving the ASO intact (Figure I.2) (21). Another ASO mechanism include translational arrest by steric hindrance of ribosomal activity, interference with mRNA maturation by inhibiting splicing and destabilization of pre-mRNA in the nucleus (22).

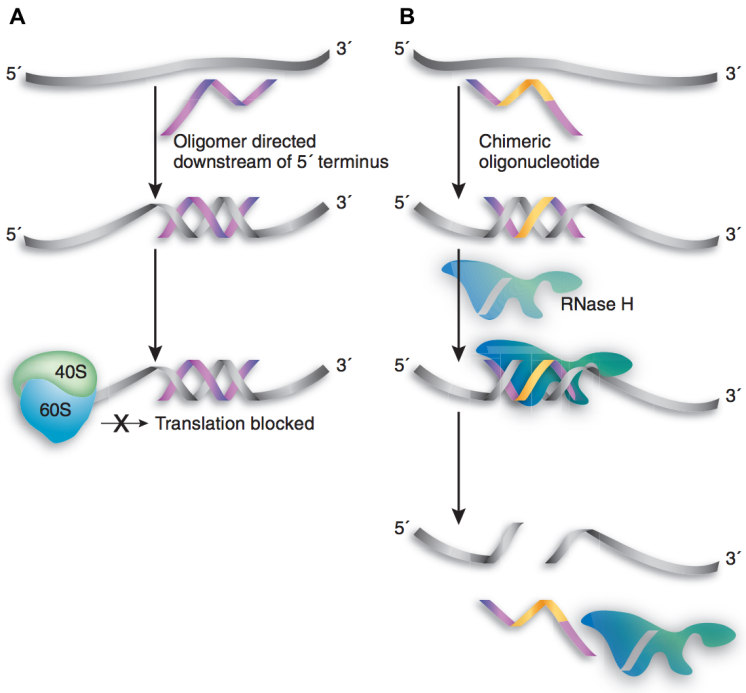


FIGURE I.2. Antisense Oligonucleotides Mechanism. (A) Steric hindrance for inhibiting gene expression through an antisense oligomer that binds mRNA and blocks translation. (B) Binding of a DNA-RNA hybrid oligonucleotide that is recognized by RNase H, which leads to the mRNA cleavage. Adapted from (23).

Since ASO first use, many chemical modifications have been made to improve resistance to nucleases and consequently, to improve their function and to limit their toxicity. One of the most successful modifications was the substitution of ribose moieties by a morpholine ring. Morpholinos, also known as phosphorodiamidate morpholino oligos (PMOs), normally consist of a 25 nucleotides chain with high specificity, water solubility and resistance to a wide range of nucleases (24,25). Morpholinos do not freely cross the cells' membrane, their entry in the cell is mediated by endocytosis and need to be aided by a delivery mechanism, either electroporation, conjugation with a weakly-basic polyamine (ethoxylated polyethylenimine (EPEI)) or the commercially available agent Endo-Porter® (26). Due to this fact, Morpholinos cannot be used in *in vivo* experiments. In order to circumvent this problem, Morpholino molecule have been attached to a transporter, resulting in a dendritic structure called vivo-morpholino that allows an effective cell membrane penetration (27,28).

Locked nucleic acids (LNAs) are another type of oligonucleotides widely used. In LNAs a modified ribonucleotide is used, where the ribose moiety presents an extra bond between the 2'-oxygen and the 4'-carbon. As such, LNAs present a rigid structure with resemblance with RNA, high affinity with complementary RNA as well as ssDNA, high specificity toward the target, high stability *in vivo*, lack of toxicity and good resistance to degradation by 3'-exonucleases. In addition, it is possible to synthetize oligomers with lower sizes (6 to 20 nucleotides) and with varied ratios of LNA, allowing different mixtures like LNA/LNA, LNA/DNA and LNA/RNA. LNAs act mainly by recruiting RNase H to degrade the aberrant mRNA, although non-RNase H based mechanisms have also been reported (29).

Currently, the ASOs Fomivirsen (Vitravene® from ISIS Pharmaceuticals) have been approved for the treatment of cytomegalovirus infection and homozygous familial hypercholesterolemia, respectively. Other ASOs are already in phase 2 and 3 of clinical trials (30). These advances in antisense therapy are encouraging and contributing to the use of ASO in metabolic disorders.

1.1.3. PHARMACOLOGICAL CHAPERONES AND PROTEOSTASIS REGULATORS

Pharmacological Chaperones (PC) and Proteostasis Regulators (PR) are two classes of distinct small molecules that act directly or indirectly to promote the stabilization of nonfuncional or partial functional unstable proteins (Figure I.3), originated by specific mutations.

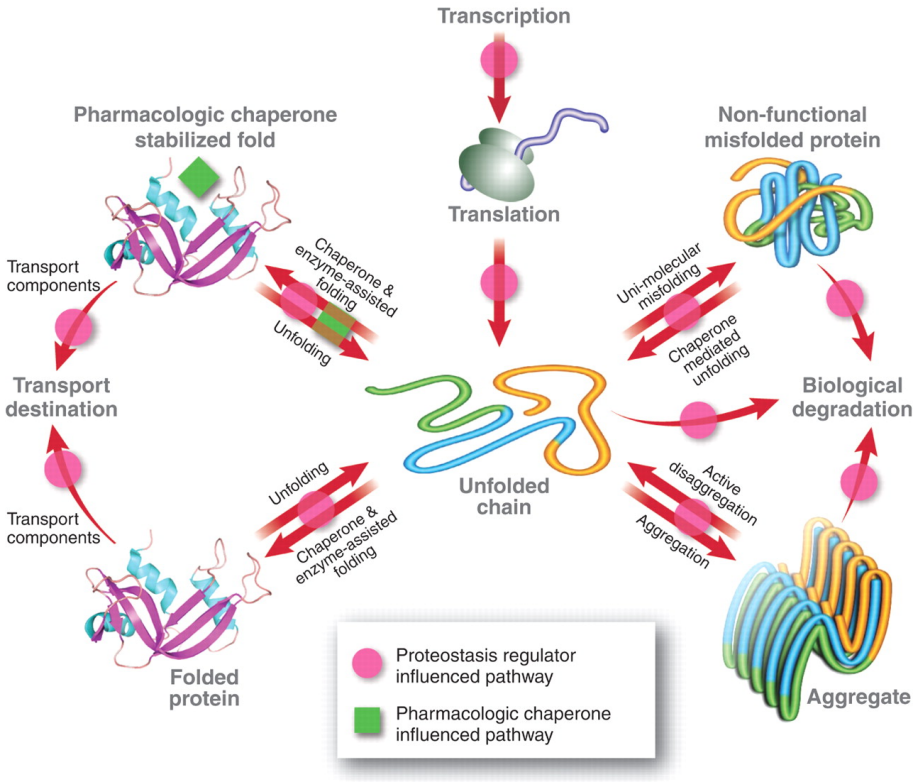


FIGURE I.3. Influence of pharmacological chaperones (green squares) and proteostasis regulators (magenta dots) in protein homeostasis pathways (red arrows). Proteostasis regulators can restore protein homeostasis and ameliorate conformational diseases through manipulation of proteostasis network, while pharmacological chaperones assist and enhance protein folding through a distinct mechanism from the innate biological pathways. Adapated from (31).

Pharmacologic chaperones are small molecules which bind to and stabilize intermediate folded states of a specific misfolding-prone protein, thereby increasing the concentration of native-like states of the mutant protein resulting in increased function or proceed to its destination environment to resume its function (32). It is considered a heterogeneous group of small compounds as it comprises molecules that bind weakly to a specific target protein, competitive inhibitors, ligands, agonists/antagonists and protein cofactors including metal ions. Interestingly, interactions with cofactors, either covalent or non-covalent bonds, are among those that contribute significantly to the maintenance of the tertiary structure of a protein (33). The downside of this approach is the small molecules specificity as they have to be tailored for each non-homologous protein linked to a loss-of-function misfolding disease (32). Currently, PC have been developed for several inherited metabolic disorders such as Gaucher disease (34), infantile Batten disease (35) and Fabry disease (36), which means that they have been adopted as a therapeutic strategy to ameliorate protein misfolding diseases.

When it comes to small molecules to rescue folding defects in proteins, the term chemical

chaperone often arises. The slight difference between chemical and pharmacological chaperones comes down to the unspecific action for the former and the more specific direct action, over a particular target protein, observed in the latter (33). Chemical chaperones are mainly osmolytes and include polyols (e.g. glycerol, sorbitol), sugars (e.g. trehalose), methylamines (e.g. trimethylamine-N-oxide), free amino acids (glycine, taurin) or its derivatives (e.g. ectoine and gama-aminobutyric acid) and also other low molecular weight compounds with a chaperone-like function, such as dimethyl sulfoxide (DMSO) (33). As mentioned before, chemical chaperones have an unspecific mode of action and do not bind directly to proteins, their stabilization action results from the hydration effect which results from the ability of water molecules to establish favourable interactions with polar groups from the protein backbone, thus increasing protein compactness (37,38). Chemical chaperones had shown being effective in rescuing misfolding proteins in diseases such as cystic fibrosis, characterized by the impairment of a transmembranar protein (cystic fibrosis membrane conductance regulator (39), and also in several mild forms of phenylketonuria (40,41).

Proteostasis Regulatores act on the machinery responsible for maintaining the cellular protein homeostasis (proteostasis) including molecular chaperones among other components. Molecular chaperones are defined as any protein that interacts with and aids in the folding or assembly of another protein without being part of its final structure (42). They are central elements of the proteostasis network (PN), which is considered a fully integrated, layered system, unique to each cell type/compartment (43), comprising nearly 1000 molecular chaperones. Molecular chaperones regulate protein synthesis, folding, trafficking, disaggregation and degradation (32). The PN is used by cells to respond to proteome insults through stress sensors and inducible pathways which include the heat shock response (HSR), the unfolded protein response (UPR), oxidative stress pathways and growth factor and diet sensitive pathways (44–46). For this reason, molecular chaperones are designated as stress proteins or heat shock proteins (Hsp) and were initially named according to their molecular weight monomers (Hsp40, Hsp60, Hsp70, Hsp90, Hsp110 and small Hsp), although most of them exist as oligomers. Based on the sort of interaction with client proteins, molecular chaperones are also classified in holdases, foldases, and disaggregases (47). Foldases and disaggregases are molecular chaperones that function broadly in *de novo* folding and refolding (i.e. the chaperonins, Hsp70s and Hsp90s). They are ATP regulated and recognize segments of exposed hydrophobic amino acid residues, which are later buried in the interior of the natively folded protein. Binding to hydrophobic segments enables these chaperones to recognize the non-native states of many different proteins. Folding is then promoted during ATP- and cochaperone-regulated cycles of binding and release of non-native protein. In this mechanism of kinetic partitioning, (re)binding to chaperones blocks aggregation and reduces the concentration of free folding intermediates, whereas transient release of bound

hydrophobic regions is necessary for folding to proceed. Holdades are ATP-independent chaperones (such as the small Hsps) that buffer aggregation. They can recognize and stabilize partially folded proteins, preventing their aggregation and presenting client proteins to foldases (47–49).

Because protein molecules are highly dynamic, constant chaperone surveillance is required to ensure proteostasis. Recent advances suggest that unbalanced cell proteostasis are in the origin of aging and also of pathological states related to a wide range of diseases such as oncological diseases, Alzheimer's, Parkinson's and metabolic disorders. Presently, the Hsp90 inhibitor 17-allylaminogeldanamycin (17-AAG) is the first modulator to enter clinical trials (50) due to evidences of recent work that identified the molecular chaperone Hsp90 as being involved in the stabilization and conformal maturation of many signaling proteins that are deregulated in cancers, showing that its inhibition results in the proteosomal degradation of these client proteins and consequently leads a possible antitumor activity (51). In addition, celastrol and MG-132, heat shock transcription factor 1 (HSF-1) enhancers, have been described to increase cytosol's proteostasis capacity and might induce one or more of the three pathways of the unfolded protein response (UPR) that remodels the proteostasis network and were stated to be candidates to treat two loss of function diseases (52).

Hence, while a decline of the PN is detrimental to cell and organismal health, a controlled perturbation of this network may offer new therapeutic avenues against human diseases (53,54).

1.2. NON-MUTATION SPECIFIC THERAPY

The development of strategies where mutation nature is not considered has also showed very interesting outcomes in a wide range of genetic diseases, including metabolic disorders, neurodegenerative, haematological, immunological and ocular. This type of therapeutic approach is considered transversal and effective against different type of genetic diseases, for which the gene causing the underlying condition has been identified, regardless how well the underlying pathophysiology is understood. Hence, non-mutation specific therapies emerge as a promise of providing lasting therapies and even cures for diseases that were previously untreatable.

1.1.1. GENE THERAPY

Gene therapy is the delivery of genetic material into an individual's cells and tissues to treat inherited or acquired diseases. It is seen as a promising molecular approach that directly delivers into the host cell nucleus a gene aimed to repair the genetic defect, in order to cure or ameliorate the clinical phenotype (55,56). Although, gene therapy can provide treatment for

complex genetic diseases and acquired genetic diseases, its ideal target are monogenic diseases (56,57). According to literature, gene therapy has been shown to be effective in treating metabolic diseases (58), immune-deficiencies (59,60), eye (61) and coagulation disorders (62). These type of disorders are characterized by the dysfunction of a single specific gene, where the complete absence or the reduction in activity of the gene product causes the pathological phenotype. Thus, the introduction of one or more copies of the healthy gene is able to restore the genetic defect (63).

A number of methods have been stablished to accomplish gene delivery, taking into account not only the characteristics of the different genetic diseases, such as the size of therapeutic gene to be transferred and the tissue affected, but also several characteristics of what is an ideal vector: efficient and specific transduction of the target cell regardless of cell cycle, a therapeutic level and proper duration of gene expression, no associated genotoxicity, absent pre-existing immunity against the vector and transgene and a non-invasive delivery route (58). Effective strategies for clinical gene therapy are based in either *in vivo* or *ex vivo* gene delivery methods. The *in vivo* method involves direct introduction of the vector, carrying the therapeutic gene, into the patient, either into or near the target organ. Although this procedure might lead to inadvertent gene transfer into tissues and cell types that are not proper targets and may elicit immune responses towards the transgene and the vector or even damage to healthy genes, it may be ideal for metabolic diseases for which liver transplantation is a treatment (64). The *ex vivo* gene therapy implies the isolation of target cells, from donors or patients, to be genetically modified *in vitro*. Cells are harvested and transduced with a vector to express the therapeutic gene at normal or even supra-normal levels. Subsequently, to restore the healthy phenotype, the gene-corrected cells are infused into the patient where they can proliferate (57). This approach is appropriate for tissue specific diseases for which cell expansion or transplantation have been stablished as a treatment and for this reason has largely been focused on genetic diseases that involved hematopoetic derived cells (65,66).

Different types of gene delivery systems have been exploited as useful vehicles for gene delivery: viral and non-viral vectors. The most common viral vectors are derived from pathogenic viruses: retroviruses, lentiviruses, adenoviruses and adeno-associated viruses (58). Actually, viral vectors arise from its innate ability of deliver genetic material into infected cells. The principles for the generation of viral vectors are the elimination of the viral toxic and infective functions, without altering the capacity to efficiently infect cells and deliver new transgenes (67).

Non-viral vectors, when compared with viral vectors, tend to be less immunogenic, to have larger packing capacity and easier to be produced. However, the cellular uptake is not very efficient. Several strategies, such as the use of polymeric systems and compounds or physical delivery methods have been used in order to overcome non-viral vectors limitations. Those

efforts may, in the future, result in a shift from the use of viral delivery to the use of non-viral delivery, but, in the present, viral delivery is the preferred approach in gene therapy(58).
In fact, new vectors are constantly under development with the aim of targeted integration and DNA editing. At the beginning, early clinical trials for gene therapy were initiated with the hope that a new era for the treatment of inherited diseases would begin, but, only more recently, gene therapy clinical trials have demonstrated safety and efficacy in treating genetic diseases. Gene therapy is certain to represent a new and exciting therapeutic option, when one considers that there are no or inadequate treatment for a majority of patients with genetic diseases (58).

1.1.2. ENZYME REPLACEMENT THERAPY

Enzyme Replacement Therapy (ERT) is a medical treatment to reintroduce an enzyme into a patient who has deficiency of a specific enzyme, and is usually associated with inherited metabolic diseases. The concept of systemic delivery of an enzyme, to rescue cellular function, first started with Lysosomal Storage Diseases (LSD) and it derives from early cell-culture experiments by Neufeld and her group (63,68). ERT is usually performed by infusions of an enzyme that is purified from human or animal tissue or blood or produced by novel recombinant techniques. Typically, the enzyme is modified to allow for a longer half-life, more potent activity, resistance to degradation or targeting to a specific organ, tissue or cell type (69). One of the first successful ERT was for alpha-1-antitrypsin (A1AT) deficiency using derived purified human A1AT (70) and the second one was developed for type I Gaucher disease, an inherited deficiency of the lysosomal enzyme β-glucocerebrosidase that leads to accumulation of the substrate in lysosomes. Initial ERT for Gaucher disease Type I used highly purified placenta-derived glucocerebrosidase and later on recombinant technology, constituting now the standard therapy for this disease (71).
Currently, ERT is also available to treat other enzyme deficiency syndromes such as Fabry disease, Pompe disease, Hurler and Hunter syndrome, lysosomal acid lipase deficiency and several of the rarer forms of mucopolysaccharidoses (69).
Despite the advances in ERT, several technological constraints limit the use of ERT as protein formulation due to the inherent instability of these macromolecules. Besides that, no enzyme given intravenously crosses the blood-brain barrier, which makes ERT inappropriate for metabolic diseases that affects the brain (72).
Emerging strategies to mitigate ERT limitations include the use of immune tolerance regimens (73), covalent PEG attachment strategies (74), modified targeting procedures or complementary therapeutic methods, such as those involving pharmacologic chaperones or substrate reducing agents (75,76).

2. PHENYLKETONURIA

Phenylketonuria (PKU; OMIM 211600), the most common inborn error of amino acid metabolism, is an autosomal recessive disorder caused by a deficiency of the hepatic human phenylalanine hydroxylase enzyme (hPAH; EC 1.14.16.1) (77). PKU was first described by Asbjørn Følling, one of the first Norwegian physicians to apply chemical methods to the study of medicine (78). Nowadays it is known as a worldwide genetic disease with marked regional and ethnic variation in its incidence, with a reported prevalence of 1 in 10,000 live births in Caucasians (77).

The deficiency in hPAH causes high levels of L-phenylalanine (L-Phe) concentration in plasma and, if left untreated, this condition is accompanied by progressive mental retardation, neurological and behavioral problems due to the neurotoxic effect of hyperphenylalaninemia (HPA) (77). To date, more than 600 disease causing mutations have been identified in the *PAH* gene (see PAH Mutation Analysis Consortium database; http://www.pahdb.mcgill.ca/) and most of them are associated with PKU (79).

The discovery of PKU by Dr Asbjørn Følling was an important milestone in medicine as the PKU model was, since then, used to link neurological effects to metabolic abnormalities and has also been used as a template to shed light on over 200 other IEM (80).

2.1. METABOLIC PATHWAY AND CLINICAL PHENOTYPE

The amino acid Phe exists as D and L enantiomers, being L-Phe one of the essential amino acid required for protein synthesis in humans, once is not synthesized endogenously (81). Dietary intake of L-Phe along with endogenous recycling of amino acid stores are the main sources of Phe, whereas, in the cell, the consumption of L-Phe occurs via integration into proteins, oxidation to L-Tyrosine (L-Tyr) or conversion into other metabolites (Figure I.4) (80). The initial and rate-limiting step in the complete catabolism of L-Phe to CO_2 and water is its hydroxylation to L-Tyr that occurs mainly in the liver. This reaction is catalysed by the complex Phe hydroxylating system, consisting of hPAH, the pterin cofactor (6R)-L-erythro-5,6,7,8-tetrahydrobiopterin (BH_4), and several enzymes that serve to regenerate BH_4 (dihydropteridine reductase and 4α-carbinolamine dehydratase) (80). This catabolic pathway accounts for approximately 75% or more of the disposal of dietary phenylalanine, which explains the major role of hPAH in maintaining L-Phe homeostasis (80).

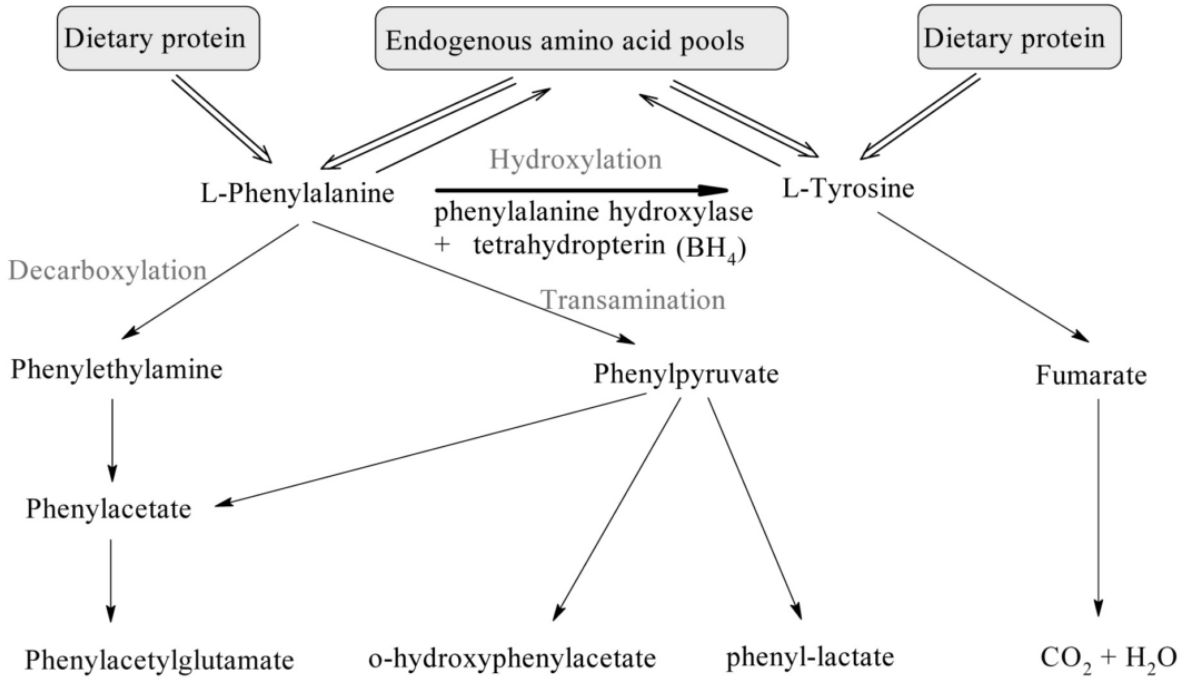


FIGURE I.4. L-Phe metabolism pathways in humans. L-Phe pool results from diet intake and from recycling trough endogenous amino acid pools from protein degradation. In the main pathway, L-Phe is hydroxylated by phenylalanine hydroxylase in the presence of its cofactor BH_4 and molecular O_2 producing L-Tyr. The alternative metabolism produces various metabolites, by decarboxilation or transamination, which are excreted in urine. Adapted from (80).

The benzene ring of L-Phe cannot be ruptured without first being hydroxylated in the para position. However, the alanine side-chain of the amino acid can be metabolized in the absence of the ring hydroxylation step. This alternative pathway is initiated by transamination or descarboxilation of L-Phe to phenylpyruvate or phenylethylamine, respectively, followed by conversion of the latter compounds to metabolites such as phenyllactate, phenylacetate and o-hydroxyphenylacetate, which are posteriorly excreted in the urine. The alternative pathway is relevant only when the cell is not able to hydroxylate the L-Phe ring in the para position (82). The compromised activity of hPAH results in the failure to convert the L-Phe into L-Tyr, both indispensable aromatic amino acids with a requirement set at 25 mg/kg per day. The hPAH deficiency results in high levels of L-Phe and low levels of L-Tyr (83). Consequently, in untreated scenery, high levels of circulating L-Phe (HPA) give rise to a spectrum of disorders (84).

Any degree of HPA could be referred as a “phenylketonuric” phenotype and would be a risk factor to be managed appropriately. According to the HPA degree, various classification schemes have emerged. For example, one proposed by Kayaalp and collaborators (85) uses a simple nomenclature where PKU is related to (i) most severe type (classical PKU) which, in an untreated state, is associated with plasma L-Phe concentrations higher than 1000 μmol/L

and dietary L-Phe tolerance of <500 mg/day; (ii) non-PKU HPA which is associated with plasma L-Phe concentrations consistently above normal (>120 μmol/L) but lower than 1000 μmol/L when an individual is on a normal diet; and (iii) variant mild group where individuals do not fit the description for either PKU or non-PKU HPA. Another classification scheme proposed by Guldberg and co-workers (86) subdivides PAH deficiency into four categories based on L-Phe tolerance before the age of 5 years. However, despite the widespread utilization of these schemes, the classification is not always straightforward because L-Phe concentrations and L-Phe tolerance are not easily and accurately measured in newborn babies. Indeed, some authors claim that there may be little value in trying to classify this disorder based on current knowledge, as there is no clear clinical application (87).

Clinically, untreated PKU children with the classical PKU phenotype, show impaired brain development. Signs and symptoms include microcephaly, epilepsy, severe intellectual disability and behaviour problems. The excretion of excessive L-Phe and its metabolites can create body odour and skin conditions such as eczema. The mechanisms by which elevated blood L-Phe concentrations disturb cerebral metabolism and cognitive function are not fully understood. However, there is a proposed mechanism that includes a potential effect of disturbed large neutral amino acid (LNAA; which includes L-Phe, L-tryptophan (L-Trp), L-Tyr, L-leucine; L- isoleucine and L-valine) transport from blood to the central nervous system (CNS) reflecting the competitive nature of the transporter for LNAA at the blood-brain barrier (BBB). The low levels of L-Trp and L-Tyr in the CNS will impact the synthesis of neurotransmitters namely serotonin and dopamine (from L-Trp) and epinephrine and norepinephrine (from L-Tyr). In addition, affected individuals have a decreased myelin formation and protein synthesis. White matter pathology will occur (88) and further problems can emerge later in life (89). In the third or fourth decade of life, individuals late- or never-treated may develop severe behavioral or psychiatric problems such as depression, anxiety and phobias (90).

Early treatment in individuals with PKU prevents severe complications as the ones described previously. However, a growing evidence suggests that individuals with strict adherence to diet may still have some sequelae and suboptimal cognitive outcome (91).

2.2. FROM GENE TO PROTEIN

2.2.1. THE *PAH* GENE AND PKU GENOTYPES

The *PAH* gene spreads over 90 kb and consists of 13 exons (representing 3% of the genomic sequence), with introns varying from 1 to 20 kb. It maps to chromosome 12, region 12q23.2. Chromosome 12 is particularly rich in disease-associated *loci*, with 487 *loci* accounting for 5.2% of known ‘‘disease genes” (77). The *locus* for the gene encoding the enzyme hPAH,

covers 1.5 Mbp and harbours five other genes of known and unknown function (International Human Genome Sequencing Consortium, 2001). The cDNA and full-length genomic sequences are both visible online in PAHdb (www.pahdb.mcgill.ca/).

As mentioned before, hundreds of disease-causing mutations in the *PAH* gene have been identified and registered in the Human PAH Mutation Knowledgebase (hPAHdb; www.pahdb.mcgill.ca/) (Scriver et al. 2003) (77). The current spectrum of PKU mutations consists of ~60% missense mutations; ~13% of deletions; ~11% of splice mutations; ~6% silent mutations; ~5% nonsense mutations and ~2% of insertions (87). Mutations have been found in all 13 exons, but the majority are localized in the 3'-half of the gene, namely between exon 5 and 12 (92). In addition, repetitive DNA sequences are quite abundant and are now seen as causes of previously unidentified large PAH gene deletions and duplications (93), as well as, the CpG dinucleotides (n51198), which are potential sites for recurrent mutation in the *PAH* gene (94).

Although mutations at the human *locus* affecting hPAH, generally, are the cause of the disease PKU or related forms of HPA, approximately 1-2% of HPA results from mutations in genes encoding for the enzymes involved in the *de novo* synthesis of BH_4 from guanosine triphosphate (GTP) and in the regeneration system of BH_4 (94).

Apparently, the HPA phenotype of the patient is determined by the position and nature of the mutation, which interfere with the activity of the PAH enzyme. However genotype-phenotype correlations may not be a robust predictor (95), because each individual has a personal genome and even those with similar mutant PAH genotypes may not have similar "PKU" phenotypes (87). Besides, hPAH, as a homomeric enzyme, exhibit interallelic complementation (IC), a phenomenon that occurs in heteroallelic states when particular combinations of two different mutant alleles, at a given *locus*, produces a less (positive IC) or a more severe (negative IC) phenotype than their homoallelic counterparts (96). Thus, IC is of particular importance in PKU, since ~75% of the patients are heteroallelic for PAH mutations, being classified as compound heterozygous, which means that they have a different mutation for each allele (94). Experimental evidences support that genotype does predict biochemical phenotype (i.e. by Phe loading tests) but does not predict clinical phenotype (i.e. occurrence of intellectual disability) (97).

2.2.2. *PAH* PROTEIN

The PAH protein belongs to a small group of enzymes, known as aromatic amino acid hydroxylases (AAAHs) which also include tyrosine hydroxylase (EC 1.14.16.2) and tryptophan hydroxylase (EC 1.14.16.4). These enzymes catalyse the hydroxylation of aromatic rings of the amino acids L-Phe, L-Tyr and L-Trp in the presence of the cofactor BH_4 (98). In addition,

for the catalytic reaction, AAAHs require molecular dioxygen (O_2) as well as a non-heme iron in the ferrous state (Fe(II)) at the active site (98).

Human PAH is a cytoplasmic enzyme present mainly in the liver, but also expressed in some extent in the kidney and epidermis (99,100). Due to its role in the degradation of L-Phe from diet, is considered a catabolic enzyme but it also provides an endogenous source of L-Tyr to the organism, converting an essential amino acid (L-Phe) into a non-essential one (L-Tyr) (101).

In terms of structure, hPAH is a homotetrameric enzyme that *in vitro* is found in equilibrium with a dimeric form. However, the tetrameric form has been considered the biological active form. Each monomer presents 452 amino acids, a molecular mass of ~52 kDa (102), adopts an α/β structure and presents three functional and structural domains: (i) an N-terminal regulatory domain (residues 1-142), containing the serine residue which is thought to be involved in activation by phosphorylation (Ser16 in hPAH); (ii) the catalytic domain (residues 143-410), containing the non-heme iron atom; and (iii) C-terminal domain (residues 411-452), which consist in a dimerization and tetramerization motif (Figure I.5). The C-terminal domain assembles to form the functional dimeric and tetrameric forms of the enzyme (94).

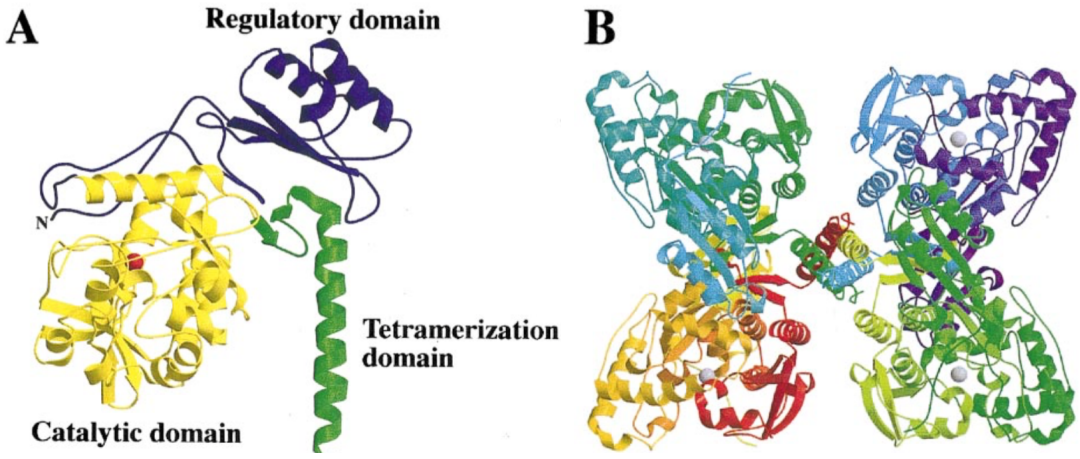


FIGURE I.5. Structure of human phenylalanine hydroxylase full-length composite model. (A) Structure of a monomer where the iron atom is shown in red; the N-terminus starting over the active site, as well as the rest of the regulatory domain is highlighted in blue; the catalytic domain in shown in yellow and the tetramerization domain is depicted in green; (B) Structure of the native tetrameric form of the enzyme. Adapted from Erlandsen and Stevens (1999) (92).

The regulation of hPAH activity is known to occur at several levels and includes allosteric activation by the substrate L-Phe, inhibition by the cofactor BH_4 and an additional activation by phosphorylation of Ser16 (103). The tetrameric form of the enzyme displays substrate activation and positive cooperativity for L-Phe binding, involving all three functional domains and all subunits (104). BH_4 acts as a negative regulator by blocking L-Phe activation when the

binding to PAH is without L-Phe forming an inactive PAH-BH_4 complex. However, recent studies demonstrate that when PAH is Phe-ativated, the BH4 binding results in positive cooperativity (105). Phosphorylation acts as a mediator of L-Phe activation by decreasing the L-Phe concentration required to activate hPAH (104).

Some of these regulatory properties are mediated by the regulatory domain. In addition, recent studies propose that L-Phe not only binds the catalytic domain but also an allosteric site localized in the hPAH regulatory domain (106,107). In fact, the hPAH regulatory domain has the ACT (Aspartate kinase, Chorismate mutase and TyrA) domain, a structural motif, common in several allosteric proteins, which is involved in the binding of small activator molecules, usually amino acids and pyrimidines. Recent studies supported an allosteric regulation of hPAH, involving the stabilization of this ACT domain upon binding of L-Phe, during hPAH activation (103). This could be an important discover to open new ways of protein activation by small molecules.

2.2.3. PKU AS CONFORMATIONAL DISEASE

The term conformational disease arises to describe disorders that are caused by the expression and/or accumulation of unfolded or misfolded proteins and to date it comprises more than 40 different conditions (108). The cell biological mechanism by which abnormal protein structures lead to conformational diseases is still unclear. However it is known that improper protein folding (misfolding) as well as accrual of unfolded proteins can lead to the formation of disorder (amorphous) or ordered (amyloid fibril) aggregates (109).

Apparently, diverse disorders can be classified as conformational diseases as their aetiology involves abnormal unfolding followed by aggregation of an underlying protein. Taking into account the pathology, proteins can be classified as: (i) naturally unfolded, proteins prone to self-aggregation due to their specific amino acid sequence; (ii) proteins that become aggregation prone only after post-translational modifications; and (3) proteins that require specific mutations to become aggregation prone (108).

A common feature of conformational diseases with gain-of-functon is that the aggregates are usually devoid of helical regions, present in the form of β-sheets and contain high percentage of post-translational modifications (109).

It is presently assumed that more than one half of all sequence alterations in genetic diseases, like PKU, are missense mutations, meaning that single amino acid substitutions can affect the structure, stability and folding of a protein turning it into a misfolded protein.

Expression and characterization of several PAH missense mutations in *in vitro* systems have identified at least three main groups of enzymatic phenotypes, which differ in their kinetic behaviour and/or stability. The first group include structurally stable mutations with altered

kinetic properties; the second group include mutations with normal or almost normal kinetic properties, but reduced stability both *in vitro* and *in vivo*; and the third group include mutations affecting both kinetic and stability properties of the enzyme(96). These mutations are located in different regions of the three-domain structures, described earlier. As, the main molecular pathogenic mechanism of PAH deficiency is protein misfolding causing protein instability and low intracellular levels, due to specific mutations, PKU is now considered a Conformational disease with loss-of-function (96,110).

For protein misfolding diseases therapeutic strategies under investigation, aim to rescue the native protein conformation or to induce stabilization of intermediate or misfolded protein states. The use of pharmacological chaperones (PC) and proteostasis regulators (PR) to rescue folding defects in proteins involved in conformational diseases have been recently explored (33,42). Actually, PC are small molecular weight compounds that usually resemble natural ligands of the target proteins and they act by stimulating protein renaturation or scaffolding the final folded structure. For example, in the case of PKU, the cofactor BH_4 is a natural ligand acting as a PC, especially when given as therapeutic supplementation (111).

There are groups that have been working on the identification of PCs in order to treat PKU (111,112). One of the groups performed a high-throughput ligand screening and, from the over 1000 pharmacological agents testes, they identified 4 compounds that improved hPAH thermal stability and did not show relevant inhibition of hPAH activity (111). The other group, developed PCs by virtual screening using BH_4 as query structure and identified 84 candidates with potential to bind the active site of hPAH (112).

Although these studies demonstrate interesting perspectives in terms of increasing hPAH activity, recently, Patel et al. disclosed the possibility to develop a new generation of PCs that specifically target the regulatory domain. This emerging model of PAH allosteric regulation, proposes that L-Phe not only bind the hPAH catalytic domain but also a site at the hPAH N-terminal regulatory domain, to activate the enzyme via an unclear mechanism (113). The synthesis of L-Phe like molecules that could act in such manner would be a stabilization strategy to target the allosteric domain and may constitute an alternative approach for the treatment of hPAH missense mutation that are non-responsive to BH_4 supplementation and that belong to the group of mutants with normal or almost normal kinetic properties, but reduced stability both *in vitro* and *in vivo.*

2.2.4. THE p.G46S MUTANT AS A MODEL TO STUDY SEVERE FORMS OF PKU

As mentioned, the majority of variant hPAH, when over-expressed in prokaryotic systems, have a propensity to self-associate and form soluble/insoluble aggregates but when produced in eukaryotic cells are rapidly degraded (114). There are no evidences about the exactly

cellular mechanism that leads to protein degradation or which are the cellular protein quality control pathways and the molecular chaperones involved in this homeostatic response in this type of variants.

The substitution of a glycine by a serine in residue 46 of the hPAH, leads to the p.G46S variant (hPAH-G46S). This protein belongs to the group of enzymatic phenotypes with reduced stability, both *in vivo* and *in vitro*, which results in a severe form of PKU (115). The p.G46S variant results from a G to A transition in cDNA position 136 (c.136g>a) of exon 2 and was first identified by fluorescence-based single-strand conformation polymorphism (F- SSCP) analysis on PKU haplotype 5.9 alleles (115). The G46 residue is located in the regulatory domain of hPAH that contains the ACT module. Residue Gly46 is located at the start of α-helix 1 (Ala47– Glu57), in a five residue (Leu41–Gly46) loop stabilized by a network of hydrogen bonds and a salt-bridge (Lys42– Glu44). It has been proposed that the G46S substitution will lead to the extension of α-helix 1 that then perturbs the α-β sandwich of the ACT module in the regulatory-domain (Figure I.6) (114). The prokaryotic expression of the hPAH G46S variant as a fusion protein coupled to the maltose binding protein (MBP) via a peptide sequence recognized by the factor Xa restriction protease (MBP-pep(FXa)-PAH-G46S), gave rise to a metastable tetrameric form characterized by a near normal catalytic efficiency (114). However, destabilization and self-association of the G46S tetramer (and dimer) and the formation of higher-order oligomers and large twisted non-amyloid fibrils were evident (Figure I.6), after the cleavage of the MBP fusion partner by the restriction protease factor Xa (114).

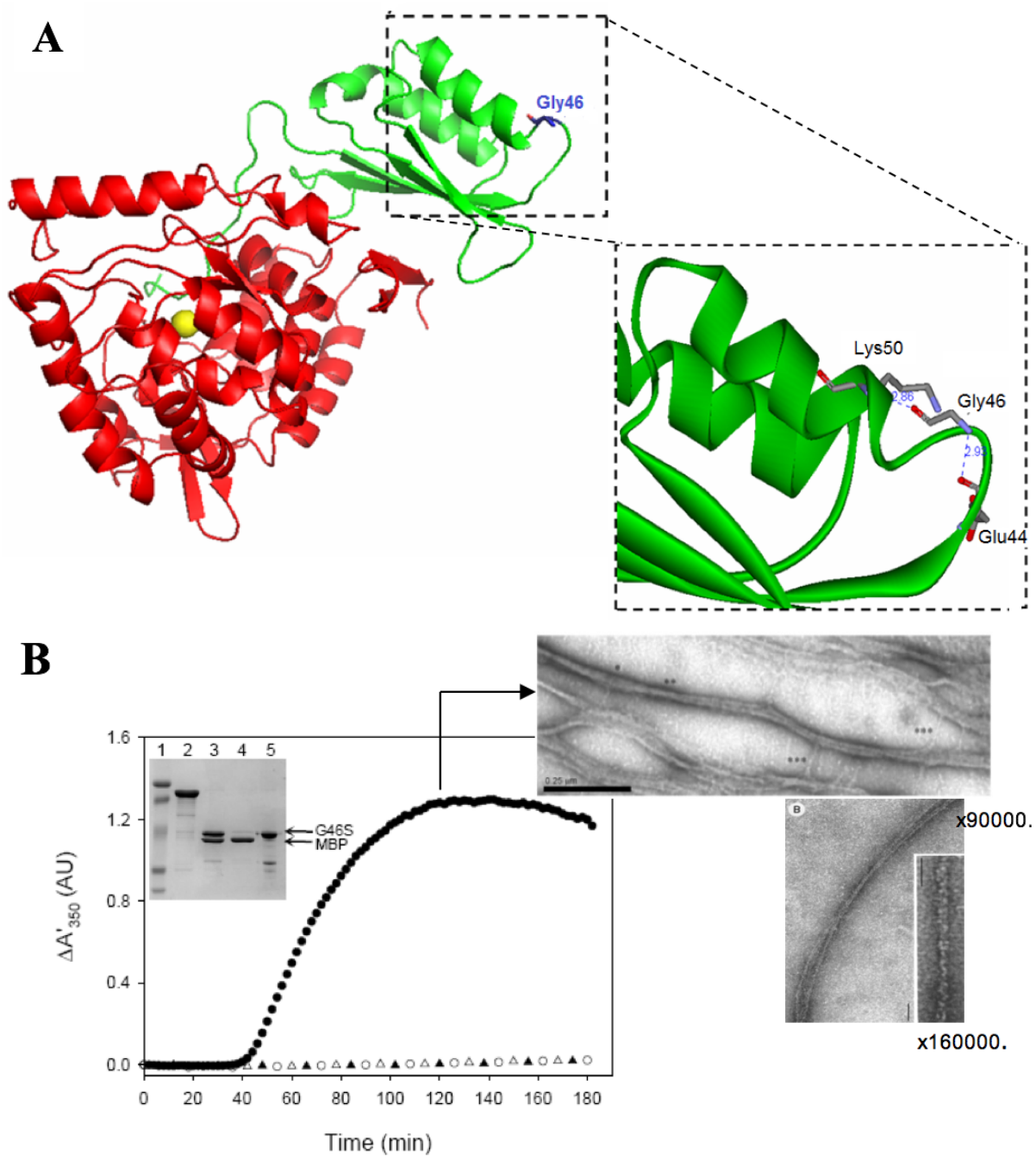


FIGURE I.6. Representation of the human phenylalanine hydroxylase monomer with the localization of residue Gly46 (A) and the self-association of the G46S tetramers (B). In panel (A) the interactions established by the Gly46 residue are shown; the regulatory domain is shown in green while the catalytic and tetramerization domains are depicted in red. Panel (B) shows the time-course of the self-association of MBP-pep(FXa)-hPAH-G46S (●) and MBP-pep(FXa)-PAH-WT (▲) following the cleavage by factor Xa; the graphic inset represents SDS-PAGE analysis of the cleavage reaction at time 0 (lane 2), 10 (lane 3), 20 (lane 4) and 30 minutes (lane 5); the electron micrographs of negatively stained hPAH-G46S at the maximum of light scattering (120 min; arrow) are also shown. Adapted from Leandro et al (114).

The variant p.G46S has been well characterized by Leandro and collaborators (114) in terms of the intrinsic physico-chemical properties, as well as extrinsic factors such as ionic strength, temperature, pH, protein concentration, phosphorylation state and deamination and it is considered a well-controlled model to study not only PR involved in protein degradation but also new PC that specifically target the regulatory domain as a stabilization strategy, with aim of to prevent self-association and to restore hPAH activity (79,96,114).

2.3. PKU TREATMENT

Currently, as there is no cure for PKU, the main goal in PKU treatment is to lowering L-Phe levels to prevent intellectual disability but maintaining the minimum levels required for normal growth. Therefore, the prevailing treatment is dietary restriction of L-Phe, supplemented with specifically designed medical foods (116).

The establishment of newborn screening programmes along with prompt institution of dietary treatment has prevented intellectual disability, although neurophysiological and neuropsychological impairments may still persist in treated patients (117–121). This dietetic restriction has, as major advantage, the applicability towards all hPAH mutations with an adequate outcome, but, as disadvantage, this strict long term diet can lead to social boundary, nutritional deficiencies (in vitamin B12, vitamin D, calcium, iron and unsaturated long chain fatty acids), which may exacerbate the neurological problems, as well as non-compliance due to poor palatability, despite the recent improvement of low-Phe dietetic products (122,123).

Therefore, there is an urgent need for exploring new approaches and alternatives to partially or totally substitute the L-Phe diet.

2.3.1. CURRENT TREATMENTS

Besides, dietary restriction of L-Phe intake, which remains the mainstay of treatment for PKU since its introduction in 1953 (124), the cofactor BH_4, LNAA and glycomacropeptide (GMP) supplementation, are the current available approaches for PKU treatment (116).

The responsiveness to BH_4 therapy is likely to be associated with mutations in the *PAH* gene resulting in some residual enzyme activity, characteristic from patients presenting the mild PKU phenotype, however genotype-phenotype correlations are inconsistent (125). Sapropterin dihydrochloride (Kuvan®, Biomarin Pharma) is a synthetic formulation of the active 6R-isomer of the naturally occurring hPAH cofactor, orally administered to patients for PKU treatment. The mechanism of action in lowering L-Phe levels, in patients with PKU, has not been fully elucidated, but appears to be related, in part, to its effect in stabilizing mutant hPAH enzymes, resulting in increased clearance of L-Phe from the body. In patients with BH_4

deficiency, its mechanism of action is presumed to be secondary to replacement of endogenous tetrahydrobiopterin (126).

Kuvan has received Orphan Drug status for the treatment of PKU, since phase II and III clinical data demonstrated that it was a safe and effective therapy (127). In fact, this therapeutic approach has proven successful in significantly increasing L-Phe tolerance allowing patients to relax their diet and in some cases discontinuing the L-Phe restrictive diet (128,129). However, for 90% of patients with classical PKU, who comprise about 50-80% of patients detected by newborn screening, this therapy has no beneficial effect (130). So, alternative treatments for which efficacy is not dependent on genetic variations in the *PAH* gene may benefit these patients.

The LNAAs had been shown to reduce CNS L-Phe concentrations and consequently, cerebral damage, despite the observed increased plasma levels. This might be due to the fact that the administered LNAAs will compete with L-Phe to cross the BBB (89). Although LNAA treatment appears to have beneficial effect on executive functioning, this approach is only suitable for adults who are not adhering to a low Phe diet (131). In addition, regarding long term outcome, clinical data using this treatment strategy are not yet available (131), requiring additional studies to prove its safety and efficacy (132).

Glycomacropeptide (GMP) is a protein derived from cheese whey, naturally low in L-Phe, rich in valine, isoleucine and threonine, that when supplemented with the essential amino acids (L-Tyr, L-Trp, arginine, cysteine and histidine) can be a useful adjunct to the L-Phe restricted diet also improving palatability (133). It has been shown that the potential benefits of having GMP included in PKU diet are reduced ureagenesis, improved protein retention and L-Phe utilization (134), decreased levels of postprandial ghrelin concentration when compared to an amino acid diet (135) and increased metabolic activity (in PKU mice) (136). From this last study, the authors conclude that the GMP diet provides a more natural and physiological low L-Phe source of intact protein as compared with the synthetic amino acid (136). Although, the present data are promising, further studies are needed to investigate the effect of the GMP diet in humans, once the majority of the studies were performed in mice. This will allow further evaluation of the safety and efficacy of GMP consumption for long term.

2.3.2. FUTURE TREATMENT STRATEGIES

All the previous mentioned data highlight the need for alternative strategies for PKU treatment. Enzyme replacement therapy with PAH-based fusion proteins and protein delivery systems, along with genetic therapy and pharmacological chaperones are some of the therapeutic strategies that have been studied in order to answer this demand.

Enzyme substitution therapy using phenylalanine ammonia lyase (PAL; E.C.4.3.1.5) has been suggested as a possible therapeutic approach for PKU. PAL is an enzyme that catalyses the

conversion of L-Phe into transcinnamic acid and insignificant amounts of ammonia, and unlike PAH, is a monomer and requires no cofactors (137). Initially, when PAL started to be administered in enteric-coated gelatine capsules to PKU patients, showed reduction in L-Phe levels but, following repeated model injections, an immune response was elicited resulting in a significant decrease in half-life (138). In addition, when administered orally, low activity of PAL in gastric secretions was found due to protease degradation (138). Later, to overcome these issues, more efficient forms of PAL have been made by site-directed mutagenesis and chemical modification with polyethylene glycol (PEG). The outcome was the maintenance of PAL specific activity, reduced immunogenicity with prolonged half-life in the PKU mouse model (139–143). This PAL formulation has started phase II clinical trials (143) and, despite the progression into phase III clinical trials, pre-clinical studies indicate that weekly injections are required to sustain a significant decrease in L-Phe levels in the PKU mice for up to one year (142). Moreover, PEG-PAL dosing regimen was gender-dependent (142).

In summary, although the positive results this therapy achieved, the route of administration would be invasive and painful to the patients and a burden to their families. It would be inherent to repeated injections, physiological stress pain, cost and risks of infection. Oral administration would be a much more convenient way of delivery, however, in general, oral delivery of enzymes have been resulting in insufficient bioavailability (138).

Approaches which use probiotics and genetically modified (GM) probiotics have gained wide attention. Probiotics are live microorganisms that confer health benefits on the host, when administered in adequate amounts (144). There are extensive reports in the literature regarding the beneficial effects of probiotics in certain conditions, such as inflammatory bowel disease (145,146). Moreover, advances in genetic engineering of probiotics have made possible to increase their beneficial effect potentially targeting a wider range of disorders. Regarding PKU, the PAL enzyme has been expressed in GM organisms and orally deliver to animal models of the disease (138,147–149). The obtained data showed a reduction of 31% in plasma L-Phe levels after one hour, a further 44% reduction after two hours and also. Furthermore, PAL contained within *E. coli* was resistant to proteolytic inactivation by intestinal enzymes *in vitro* (138). However, there are safety concerns regarding the use of *E. coli* for delivering enzymes to the gastrointestinal tract as there is a potential risk that engineered *E. coli* interacts with its wild type form present in the normal gut flora (150). So, studies were performed expressing PAL in *L. lactis* resulting in a significant reduction in L-Phe levels in treated rats. However, the animals used did not represent an orthologous model to human PKU (147). These data suggest that GM probiotics may be useful as alternative therapy for PKU and may allow PKU patients to relax their diet. However, as any of the other approaches, more studies are needed to evaluate the safety and efficacy of these GM modified probiotics in a short and long-term setting.

Regarding gene therapy, the goal is to deliver a functional *PAH* gene into the liver cells, as the activity of PAH is primarily in the liver. To accomplish that, several types of viral vectors have been examined for their potential to correct PKU either using mouse models or hepatocytes derived from these mouse models. Assays using an adenoviral vector containing the hPAH-cDNA infused into the liver through portal vein of PKU mice (151) showed a therapeutic effect, that however ceased after a few weeks. Recombinant adeno-associated virus (rAAV) vectors have gained increasing attention as they do not carry any viral genes and therefore elicit minimal immune responses. However the reduction in L-Phe levels was only noted in male PKU mice (152). Recently, the transfer of foreign genes using non-viral vectors into tissues or organs by direct injection of naked plasmid-DNA started to be achieved, leading to transgene expression and therapeutic response. This approach allows no size limitation of the DNA insert and no potential cytopathic side-effect, but the gene transfer rate and the transient transgene expression are lower (153). The use of Minicircle (MC) naked-DNA vectors is another recent explored strategy and already demonstrated that it is possible to express the murine PAH cDNA leading to normalisation of blood L-Phe levels in a PKU mouse model (154). This data showed that the use of MC-DNA may offer improved safety and efficacy as a potential genetic treatment for PKU and also liver diseases (154).

Although the described studies provide a step forward to achieve efficiency and stability of cDNA delivery, more research is required to create more robust and stable vectors before implementing gene therapy.

CHAPTER II • **AIMS**

About 1 in 800 live births are affected by inherited metabolic disorders (IMD). IMDs represent a vast, diverse and heterogeneous collection of around 700 genetic rare diseases that are a significant cause of morbility and mortality, especially in childhood. By 2015 only ≈5% of rare diseases had available pharmacological treatments, which highlight the need for the development of therapeutic options for this group of patients.

Phenylketonuria is one of the most known IMD, with more than 900 mutations described to date. When untreated, causes a wide phenotypic spectrum ranging from mild to severe psychomotor impairment due to neurotoxic accumulation of L-Phe and the low levels of L-Tyr which, in turn, affect the biosynthesis of several neurotransmitters such as catecholamines. The current treatment available consists in a life-long L-Phe-free diet initiated in the first days of life, as soon as possible after diagnosis by newborn screening programmes. The administration of KUVAN® (sapropterin dihydrochloride), a synthetic formulation of BH_4, the naturally occurring hPAH cofactor, is also an available treatment introduced very recently, due to its pharmacological chaperone-like activity. However, lack of diet compliance resulting in poor metabolic control and the non-responsiveness of KUVAN® treatment by patients with severe forms of PKU, emphasise the urgent need for new therapeutic approaches.

Recently, Leandro et al. and other groups has shown that PKU is a misfolding disease with loss of function, since the majority of the mutations result in proteins displaying disturbed oligomerization, decreased stability and accelerated degradation. A novel therapeutic strategy to ameliorate misfolding diseases relies in the adaptation of the cellular proteostasis. Therefore, small molecules acting as pharmacological chaperones and/or proteostasis regulators could be used to modulate the balance between folding and degradation, which in loss-of-function conformational diseases is compromised. In addition, despite limited application of proteins as therapeutic agents due to their complex nature and instable structure, one of the most promising therapeutic strategy to PKU is enzyme replacement therapy.

In the latest years, the *Metabolism and Genetics* (Met&Gen), *Bioorganic Chemistry* and *Nanostructured Systems for Overcoming Biological Barriers* (Nano2B) groups from the *Research Institute for Medicines* (iMed.ULisboa) have been devoted to the development of new strategies for PKU treatment either through the synthesis of small molecules acting as pharmacologigal chaperones and/or enzyme activators or through and enzymatic replacement therapy approach. Previous work of the Met&Gen group suggests that the severe p.G46S hPAH variant (associated with a severe PKU phenotype) is a useful model to be used in the systematic *in vitro* studies to assess the ability of small molecules to inhibit hPAH aggregation. In addition, the group have also been working, together with Nano2B group, in the development of an enzymatic replacement approach, where a nano-system based on

chitosan (CS) to encapsulate hPAHwt has been studied not only to protect the enzyme during formulation but also to be used as a delivery system.

The present thesis intends to continue the collaborative work that has been developed within the Met&Gen, Bioorganic Chemistry and Nano2B groups. Therefore, this project aims to:
(i) Study the effect of several small compounds synthesized by the Bioorganic Chemistry group upon the aggregation of the hPAH-G46S variant, *in vitro* and *in cellulo*, hoping to contribute to the identification of a new class of pharmacological chaperones;
(ii) Identify and validate therapeutic targets within the components of the cytosolic proteostasis network in order to develop new pharmacological options for the treatment of severe forms of PKU (such as the p.G46S variant) by using small molecules that could function as proteostasis regulators;
(iii) Evaluate the stability and activity of nanoencapsulated hPAHwt in a cellular environment, in order to validate the previously optimized delivery system.

CHAPTER III • **MATERIAL AND METHODS**

1. MATERIALS

1.1. REAGENTS

The Isopropyl β-D-1-thiogalactopyranoside (IPTG) and lysozyme were obtained from Nzytech. Ferrous ammonium sulfate was from Merck. The protease inhibitor phenyl-methylsulfonyl fluoride (PMSF), the cofactor BH_4, L-Phe, Hepes, dithiothreitol (DTT), low molecular weight CS (50-190 kDa) with a deacetylation degree of 91.8 % and tripolyphosphate (TPP) were purchased to Sigma-Aldrich. The amylose resin and the restriction protease factor Xa were obtained from New England Biolabs.

The ISOLATE II Mini Kit from Bioline was used for DNA purification. Branched polyethylenimine (PEI) 25 kDa from Sigma-Aldrich and Lipofectamine® LTX reagent from Invitrogen were used as co-transfection agents. Opti-MEM® I reduced-serum medium was obtained from Thermo Fisher.

For the cell culture assays, RPMI medium, bovine serum, penicillin and streptomycin were obtained from Invitrogen. The Magnetic Beads, used in the co-immunoprecipitation assays, was delivered by Bio-Rad.

1.2. EXPRESSION VECTORS

1.2.1. pTrcHis

The prokaryotic expression vector pTrcHis (appendix 1) was used to produce the wild-type PAH enzyme and was obtained from Invitrogen. The hPAH cDNA (GenBank U49897) had already been cloned into the BamHI site of pTrcHis vector and the expression system has been previously validated by the research group (155). The expression construct contains a sequence that encodes a hexahistidyl peptide (6xHis tag), N-terminally to the recombinant hPAH that function as a metal binding domain in the translated protein allowing purification of the recombinant protein by immobilized metal affinity chromatography (IMAC). It also contains a codifying sequence for an amino acid sequence presenting the cleavage recognition site for the protease enterokinase (EK) between the metal binding domain and the recombinant protein which allows the subsequent removal of the N-terminal fusion peptide from the purified recombinant protein.

1.2.2. pMAL-c2X

The prokaryotic expression vector pMAL-c2X (Appendix 2) was from New England Biolabs and was used to produce the recombinant hPAH-G46S. The hPAH cDNA has been already cloned according to the procedures described in (96). The hPAH-G46S cDNA was inserted downstream from the nucleotide sequence encoding the maltose binding protein (MBP) N-

terminally to the hPAH-G46S, resulting in the expression of an MBP fusion protein which can be purified by affinity chromatography using an amylose resin. The pMAL-c2X vector also contain a sequence encoding for an amino acid sequence recognized by the protease Factor Xa (FXa), located just 5' to the polylinker insertion sites, which allows MBP to be cleaved from the protein of interest after purification.

1.2.3. pEXP51

The pEXP51 vectors were used to build the molecular chaperones library. These vectors were obtained through the Gateway™ cloning technology from Invitrogen. This approach utilizes the pEF-DEST51, a destination vector for cloning and expression of C-terminal fusion proteins in mammalian cells. The Gateway technology consists in cloning the target gene in the pENTR vector in FUSION format (containing flanking attL sites, the Kozak sequence at the 5' terminus and no stop codon at the 3' terminus), and further recombine them with destination vectors (pDEST) that contain attR sites and the backbone of the final expression vector. The recombination between pENTR vector and pDEST vector result in the pEXP51 expression vector (Figure VIII.4) (156,157). Using these vectors the molecular chaperones are expressed in fusion with a N-terminal 6xHis tag.

1.3. BACTERIAL STRAINS AND EUKARYOTIC CELL LINES

The hPAH-G46S protein was expressed in *Escherichia coli* TB1 (F- ara Δ(lac-proAB) [Φ80dlac Δ(lacZ)M15] rpsL(StrR) thi hsdR) obtained from New England Biolabs (USA), while the hPAHwt protein was expressed in *Escherichia coli* TOP10 (F-, mcrA Δ (mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 recA1 araD139 (ara-leu)7697 galU galK rpsL endA1 nupG) from Invitrogen.

For the mammalian cell culture studies, three different cell lines were used namely the HEK-293T cells (human embryonic kidney epithelial cell line, ATCC CRL-11268), the HepG2 cells (human hepatocellular carcinoma cell line, ATCC HB-8065) and the COS-7 cells (CV-1 cell line with an Origin defective mutant of SV-40, ATCC CCL-70).

1.4. MOLECULAR CHAPERONES

The molecular chaperones studied in this work are presented in Table III.1 and were cloned in the appropriate eukaryotic expression vector according the Gateway technology (Invitrogen) during Met&Gen group's previous work as described in 1.3.

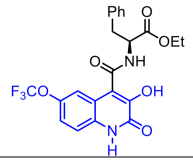
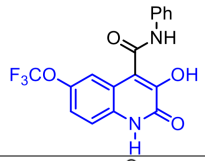
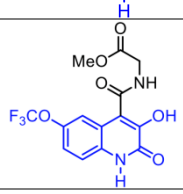
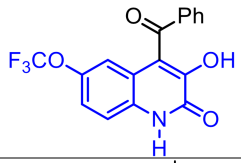
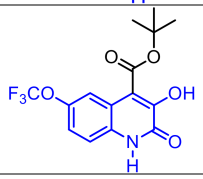
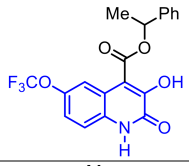
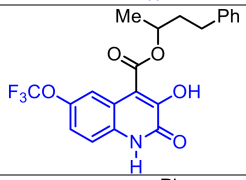
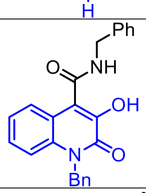
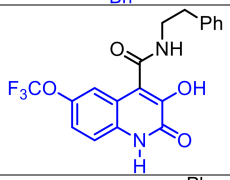
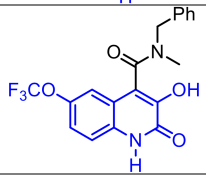
TABLE III.1. Molecular Chaperones screened for their interaction with the hPAH-G46S variant form.

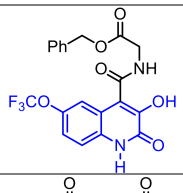
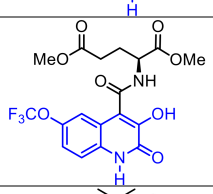
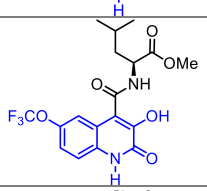
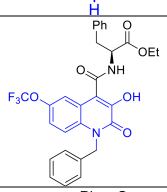
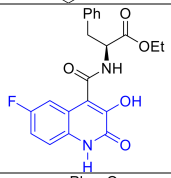
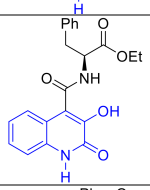
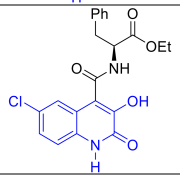
Molecular Chaperone	MM (kDa)	Expression vector	Main role	Reference
HSP90AB1 Heat shock protein HSP 90-beta UniProtKB–P08238	90	pEXP51V5/His85	Promotes maturation, structural maintenance and proper regulation of specific target proteins. Interacts dynamically with various co-chaperones that modulate its substrate recognition, ATPase cycle and chaperone function	(158)
HSPA8 Heat shock cognate UniProtKB-P11142	71	pEXP51V5/His553	Participates in ER-associated degradation (ERAD) quality control pathway.	(159)
HSPA2 Heat shock-related protein 2 UniProtKB-P54652	70	pEXP51V5/His555	Plays a pivotal role in protein quality control system, ensuring the correct folding of proteins, the re-folding of misfolded proteins and controlling the targeting of proteins for subsequent degradation.	(160)
HSPH1 Heat Shock Protein UniProtKB–Q92598	105	pEXP51V5/His556	Member of the Hsp70 super family, independently prevents the aggregation of misfolded proteins.	(161)
SQSTM1 Sequestosome-1 UniProtKB-Q13501	50	pEXP51V5/His575	Required both for the formation and autophagic degradation of polyubiquitin-containing bodies.	(100)

1.5. TESTED COMPOUNDS

A library of 17 different derivatives of 3-hydroxyquinolin-2(1H)-one (3HQ) was utilized to follow the inhibition of hPAH-G46S aggregation after FXa cleavage. These compounds were synthesized by the Bioorganic group (iMed.ULisboa) and are presented in table III.2.

TABLE III.2. Library of quinoline derivative compounds tested for inhibition of hPAH-G46S aggregation

Compound Structure	Compound Designation	Molecular Mass
	C2	464.39
	C4	364.06
	C6	360.24
	C8	349.26
	C9	345.27
	C10	393.31
	C11	421.36
	C12	384.42
	C14	392.32
	C15	392.32

	C16	436.33
	C17	446.33
	C18	416.34
	C19	554.52
	C20	398,39
	C21	380.40
	C22	414.84

2. METHODS

2.1. EXPRESSION AND PURIFICATION OF hPAH-G46S

For the expression of recombinant hPAH-G46S, 100 µL of *E. coli* TB1 competent cells were transformed with 10 ng (5 µL) of the DNA construct (pMAL-MBP-Xa-hPAH-G46S). The cells were grown overnight (ON) at 37°C, on solid LB (tryptone 1%, yeast extract 0.5%, NaCl 0.17 M, pH 7, agar-agar 1.5%) containing ampicillin (LB/Amp) at 100 µg/mL. An isolated colony was selected to inoculate 10 mL of LB/Amp medium. After ON incubation, at 37°C with constant stirring (≈110 rpm; Selecta Rotabit 3000974 orbital shaker), the bacterial suspension was diluted in 1L (1:100) of LB medium with 50 µg/mL Amp, 200 µM ferrous ammonium sulphate and 0.2% of glucose and was incubated at 37°C with constant stirring (≈160 rpm; Selecta Rotabit 3000974 orbital shaker) until reach an optical density of ≈0.6 at λ600 nm (OD_{600nm}). At this time point, the medium was supplemented with 1 mM isopropyl-thio-β-D-galactoside (IPTG) (to initiate hPAH-G46S expression) and 200 µM ferrous ammonium sulphate. After incubation during 3 h, at 28°C, at 160 rpm, a novel supplementation of 200 µM ferrous ammonium sulphate was performed and the medium was maintained at 28ºC for an extra 2h with constant stirring (160 rpm). The bacterial cell pellet was then obtained by centrifugation at 3220x*g*, during 15 minutes at 4°C and washed with cleaning buffer (20 mM KH_2PO_4, 0.15M NaCl, 1mM EDTA, 1mM PMSF, pH 7.5). The bacterial pellet was stored at -20°C for a short period (no more than three months), until being used.

In order to prepare the sample for purification, the pellet was ressuspended in MBP-lysis buffer (10 mM NaHepes, 200 mM NaCl, pH 7.4, 1 mM EDTA, 1 mM PMSF, pH 7.25) containing lysozyme (1 mg/mL) and DNAse (≈5 µg) in a ratio of 1 L of culture/10 mL of MBP-lysis buffer. After 30 minutes of incubation, at 4°C, cells were lysed by sonication (Sonics Materials - VibraCell), through 3 cycles of 60 seconds each (with a duty free cycle of 50%). Between each cycle, the lysate was maintained during 30 seconds, at 4ºC, to allow the sample cooling down. The suspension was then centrifuged at 12800x*g* centrifugation during 40 min, at 4°C, to separate the cellular residues and aggregated proteins from the soluble cytoplasmic fraction which was used to isolate the recombinant hPAH-G46S protein.

The variant protein was then purified by affinity chromatography using 60 mL of amylose resin (New England Biolabs), at 4ºC. The column was previously equilibrated with 4 volumes of 15 mM NaHepes, 200 mM NaCl, 1 mM EDTA, pH 7.4. After injection of the cell lysate, the column was washed with 12 volumes of MBP-washing buffer (15mM NaHepes, 200 mM NaCl, pH 7.4). The recombinant hPAH-G46S was eluted using MBP-elution buffer (MBP-washing buffer containing 10 mM maltose). The solvents were pumped at 0.8 mL/min.

The tetrameric form of the variant recombinant protein was further isolated by size exclusion chromatography (SEC) using a prepacked HiLoad 16/60 Superdex 200 column (1.6 cm x 60

cm) from GE Healthcare, at 4°C. The mobile phase consisted of 20 mM Na-Hepes, 200 mM NaCl, pH 7.0 (SEC buffer) and the flow rate was 0.7 mL/min. The relative molecular mass (MM) of the different oligomeric forms was estimated from a calibration curve obtained using standard proteins (from Sigma), at a concentration of 5 mg/mL each: cytochrome C (12.4 kDa), ribonuclease A (13.7 kDa), myoglobin (17.6 kDa), β-lactoglobulin (18.4 kDa), ovalbumin (45 kDa), BSA (66 and 132 kDa), alcohol dehydrogenase (150 kDa), β-amylase (200 kDa) and apoferritin (443 kDa).

The tetrameric hPAH fusion protein was collected and concentrated by Amicon Ultra 15 centrifugal filter device (50 kDa MWCO; Millipore) until reaching a concentration of ≈ 9 mg/mL. The amylose affinity purification and the SEC were performed on a FPLC (Fast Protein Liquid Chromatography) system, namely the AKTA Prime Plus (GE Healthcare), equipped with a UV detector (λ280 nm) and a fraction collector.

2.2. EXPRESSION AND PURIFICATION OF hPAHWT

For the expression of the hPAHwt, 100 µL of *E. coli* TOP 10 competent cells were transformed with 10 ng (5 µL) of the appropriate DNA construct (pTrcHis-hPAHwt) to produce the hexa-histidyl hPAH fusion protein.

An isolated colony was used to inoculate 10 mL of LB/Amp medium. After ON incubation, at 37ºC, with constant stirring at 110 rpm, the medium was used to inoculate 1L of LB/Amp medium and cells were grown at 37°C until reach an OD_{600nm} ≈0.6. The expression of the recombinant hPAHwt was induced by the addition of 1 mM IPTG. At this time point 0.2 mM ferrous ammonium sulphate was also added to the culture medium. After 3h incubation, at 37°C, with constant stirring (160 rpm) bacterial cells were harvested by centrifugation at 3220*xg*, during 15 min, at 4°C. The bacterial pellet was stored at -20°C for a short period (no more than three months), until being used.

For purification, the bacterial pellet was ressuspended in 6xHis lysis buffer (50 mM KH_2PO_4/K_2HPO_4, pH 7.8, 300 mM NaCl, 10% de glycerol) containing lysozyme (1 mg/mL), PMSF (1 mM) and DNAse (≈ 5 µg) in a ratio of 1 L of culture/10 mL of 6xHis lysis buffer. After 30 min of incubation, at 4°C, cells were lysed by sonication as described previously. The cell lysate was centrifuged at 12800x*g*, during 40 min, at 4°C, to separate the cellular residues and aggregated insoluble proteins from the soluble protein fraction.

The soluble fraction was used to purify the recombinant hPAHwt fusion protein by IMAC in a ratio of 10 mL lysate/500 µL resin. Before being used the Ni-NTA resin was washed 3x with 1 mL of Milli-Q water and 3x with 1 mL of 6xHis-lysis buffer containing 10 mM imidazole. Between each step of washing and conditioning, the suspension was centrifuged at 360x*g*, during 2 min, to remove the supernatant. The Ni-NTA resin was then incubated with the

soluble fraction (previously supplemented with imidazole and β-mercaptoethanol at a final concentration of 10mM each) during 1h, at 4°C, with constant stirring. The suspension was then packed into a 9 cm polypropylene conical column (Poly-Prep columns; BioRad), and purification was performed, at 4 °C, using the 6xHis-lysis buffer containing increased concentrations of imidazole solutions (gradient): (i) washing steps of 2x 5 mL 20 mM imidazole , 2x 5 mL 50 mM imidazole and 1x 5 mL 75 mM imidazole; (ii) elution step 4x 500 μL 250 mM imidazole.

In order to isolate the oligomeric forms of hPAHwt, the FPLC system previously described was used The tetrameric fusion protein was concentrated using an Amicon Ultra 15 centrifugal filter (50 kDa MWCO), until reaching a concentration of ≈ 9 mg/mL.

2.3. SDS-PAGE PROTEIN ANALYSIS

When required, either the variant or the hPAHwt protein were analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) according to the Laemmli's method [9]. For the resolving gel, a 10% acrylamide/bis-acrylamide solution (30% T and 2.6% C; Bio-Rad) and the 0.375 mM Tris-HCl, pH 8.8, 0.1% SDS buffer was used. The stacking gel contained 4% acrylamide/bis-acrylamide and the 0.125 mM Tris-HCl, pH 6.8, 0.1% SDS buffer. The electrophoresis buffer contained 62.5 mM Tris, 192 mM Glycine, 0.1% SDS and pH 8.3. The samples were applied to the system after being denatured at 95°C, during 10 min, in loading buffer solution (62.5 mM Tris-HCl, pH 6.8, 20% glycerol, 2% SDS, 0.025% bromophenol blue and 5% β–mercaptoethanol). The electrophoresis was performed on the vertical system Mini-PROTEAN® Tetra handcast (Bio-Rad). To induce electrophoretic separation, a constant current of 20 mA was applied. After electrophoresis proteins were visualized using the BlueSafe (Nzytech) dye.

The molecular masses of the proteins were estimated by comparison with the electrophoretic profile of NzyColour protein marker II (Nzytech), a ready-to-use mixture of 12 highly purified pre-stained proteins, covering a wide range of molecular weights from 11 to 245 kDa.

2.4. PROTEIN QUANTIFICATION (BRADFORD/BCA METHODS)

The concentration of purified fusion protein was measured by two different colorimetric methods namely the Bradford and the Bicinchoninic acid (BCA) assays, and solutions of bovine serum albumin (BSA) as the standards. For the Bradford assay (163) the Bio-Rad Protein assay reagent (Bio-Rad) was used. The final volume of the reaction was 1 mL and absorbance were measured at λ595 nm in a spectrophotometer Shimadzu (UV1800) using as standards BSA solutions from 1 to 10 μg/mL. The BCA assay (164) was performed using the

BCA reagent (Sigma) and the absorbance were measured at λ562 nm in a microplate reader FLUOstar Omega (BMG LABTECH). All the assays were performed in triplicate.
The BCA assay was used to quantify indirectly the percentage of protein encapsulation, through determination of the protein concentration in the supernatant after nanoparticle deposition, as any of the formulation compounds is not a method interferer.

2.5. FXa INHIBITION ASSAY

The FXa inhibition assay was performed in order to rule out compounds that have the ability to inhibit/inactivate the FXa protease. Changes in the cleavage rate of a synthetic substrate of FXa in the presence of the compounds was measured in real-time through 60 measurements during 30 min by fluorescence spectroscopy using the FLUOstar Omega microplate reader. The assay was performed in a 96 well plate. The final volume of the reaction was 200 μL and contained the reaction buffer (20 mM Na-Hepes, 100 mM NaCl, pH 7.0), 5 μg/mL of Factor Xa, 50 μM of the substrate Z–Gly–Gly–Arg–AMC (I-1140; Bachem), and in the presence of 100 μM of 3-HQs derivatives (1% DMSO). Reaction was performed at 30°C, using λ_{exc}360 nm and λ_{em}460 nm. Appropriate controls were performed using substrate alone with 1% DMSO (Negative Control) and enzyme with no compounds and 1% DMSO (Positive Control).

2.6. hPAH-G46S AGGREGATION ASSAY

In order to study the influence of the 3HQs derivatives on the self-association process of hPAH G46S, cleavage of the purified MBP-hPAH-G46S protein by FXa in the presence and absence of the compounds was performed. The fusion protein (0.74 mg/mL) was incubated, at 30°C, in a reaction mixture containing 20 mM Na-Hepes, 100 mM NaCl, pH 7.0, factor Xa (at a final ratio (by weight) of 1:150 relative to the fusion protein) and 100 μM (1% DMSO) of tested compounds. Before the assay, the protein was centrifuged, at 2000 x*g*, for 15 min, at 4°C. The aggregation profile was followed in real-time by light scattering, measured by the increase in apparent absorbance at λ350 nm on a FLUOstar Omega microplate reader. The reaction was followed during 180 min with shaking before each measurement. The data was fitted to a plateau followed by one phase association equation:

$$y = y_0 + (Plateau - y_0) \times (1 - e^{-k(x - x_0)}) \qquad \text{Equation II.1}$$

where x_0 is the time at which the increase in absorbance begins (lag phase), y_0 is the average absorbance at time x_0, plateau is the y value at infinite time and k is the rate constant.

2.7. NANOPARTICLES PREPARATION

The particulate system was obtained by ionic gelation using a slight modification of a previously described method (165,166). Chitosan (CS) stock solution was prepared at 10 mg/mL in 1% (v/v) acetic acid and tripolyphosphate (TPP) stock solution at 10 mg/mL in MilliQ water. Stock solutions were individually diluted to the desired concentrations in SEC buffer and blank nanoparticles (NP) were instantly formed when TPP was added to CS at a fixed 1:5 (TPP:CS) volume ratio.

For the preparation of protein loaded CS NP, isolated hPAHwt tetramers in a solution of 250 μg/mL were incubated with TPP solution at concentrations suitable to maintain the volume ratios of the blank nanoparticle formulations. The hPAHwt concentration had been optimized through previous work (167). The pH of the CS solution with concentration of 1 mg/mL was also studied and indicated that must be adjusted to 5.5. The nanoparticles were instantly formed when TPP + protein solution was added to CS.

2.8. NANOPARTICLES CHARACTERIZATION

2.8.1. PARTICLE SIZE AND ZETA POTENTIAL

The mean particle size and polydispersity index (PI) were determined by photon correlation spectroscopy on a Zetasizer Nano-S (Malvern Instruments) and zeta potential was measured through laser Doppler anemometry on a Zetasizer Nano-Z (Malvern Instruments). Samples were diluted in filtered MilliQ water (0.45 μm filter; Millipore). In all cases, mean values were obtained from the analysis of three different batches, each of them measured three times. Results were expressed as mean ± standard deviation (SD).

2.8.2. ENCAPSULATION EFFICIENCY

Nanoparticle production yield was determined using an indirect method based on the quantification of the protein present in the supernatant after nanoparticle deposition by centrifugation at 40000x*g*, for 30 min, at 4ºC. The amount of protein present in the supernatant was determined using the BCA assay. All the samples were analysed in triplicate. The encapsulation efficiency (EE) was determined using the equation :

$$\mathrm{EE}\ (\%) = \frac{[\mathrm{hPAH}]_{\mathrm{total}} - [\mathrm{hPAH}]_{\mathrm{sob}}}{[\mathrm{hPAH}]_{\mathrm{total}}} \times 100 \qquad \text{Equation II.2}$$

where $[hPAH]_{total}$ represents the total amount of hPAHwt added to each sample and $[hPAH]_{sob}$ the amount of hPAH present in the supernatant after NP deposition. Thus, EE corresponds to percentage of total protein that was encapsulated.

2.9. EUKARYOTIC CELL CULTURE

The HEK293T and COS-7 cell lines were used to study hPAH-G46S and molecular chaperone interactions. The HepG2 cells were used for the evaluation of hPAHwt loaded NP. Cell cultures were grown at 37ºC with 5% CO_2 in a humidified atmosphere in RPMI medium (Invitrogen) supplemented with 10% (v/v) of heat inactivated bovine serum (60ºC for 30 min) (FBS, Invitrogen), 100 U/ml penicillin, and 0.1 mg/mL streptomycin (Invitrogen) at a suitable concentration for cells to grow exponentially during the time of the assay.

2.9.1. CELLULAR UPTAKE OF NP

In the day before the assay, 5 mL of cell culture medium ($2x10^5$ cells/mL) were inoculated in T-flasks of 25 cm^2 and incubated at 37ºC, with humidity control and 5% CO_2 (Nuaire NU4750E). In the next day, culture medium was removed and 1 mL of fresh medium plus 500 µL of hPAHwt NP formulation was added. After 1, 4, 6 and 16h of incubation, from each T-flask the culture medium supernatant was collected. The cells were trypsinized with 500 µL of TripLE Express (Dissociation reagent, Life Technologies), centrifuged for 5 min, 100x*g*, at 4ºC, and washed with cold PBS buffer (10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4). The pellet was ressuspended in 100 µL of PBS buffer with 200 µM PMSF and cells were disrupted by passing the lysate repeatedly (≈ 20 times) through a fine needle. An aliquot of this suspension was stored and referred as the whole cell lysate. After centrifugation at 12800xg, for 10 min, at 4ºC, a lysate pellet and the lysate supernatant, constituting the intracellular medium, were obtained. Together, the (i) culture medium supernatant, the (ii) whole cell lysate, the (iii) lysate pellet and the (iv) lysate supernatant constitute the four samples that were further analysed by Western Blot after SDS-PAGE separation.

2.9.2. TRANSFECTION ASSAYS

A preliminary assay was made in order to optimize the ratio between pDNA and transfectant agent for hPAH-G46S expression in eukaryotic cells. For this, cells grown in 24-multiwell plates (Greiner-Bio One) to approximately 50-60% confluence, were transfected using branched PEI (25 kDa) at 1mg/mL in a 1:6 pDNA (pDNA hPAH-G46S) to PEI ratio (w/w). For controls assays, transfection with pGFP (Invitrogen) plasmid, using the same DNA amount and DNA to PEI ratio, and cells only with culture medium were also performed. After 14 h of transfection, the medium was changed and 48 hours post-transfection hPAH-G46S expression was detected by immunocytochemistry.

2.9.2.1. MOLECULAR CHAPERONES

To prepare pDNA of the different molecular chaperones, *E. coli* TOP 10 competent cells were transformed according to standard methods and the DNA was prepared using the BioLine ISOLATE II Plasmid Mini Kit. For DNA quantification NanoDrop instrument (Thermo Scientific) was used and fluorescent signal was measured at λ520 nm on a FLUOstar® Omega microplate reader.

The HEK293T and COS-7 cells were seeded in 6-well plates and co-transfected with total amount of 750 ng DNA (375 µg for each construct: pEF-DEST51-G46S+STOP or pEF-DEST51-molecular chaperone target) per $2x10^5$ cells. Two transfectant agents were tested: PEI and Lipofectamine® LTX in a 1:6 pDNA to transfectant agent ratio (w/w). The right amount of pDNA and transfectant agent in a 1:6 ratio and 500 µL of cell medium base was mixed together and incubated for 30 min at room temperature (RT), before being added to cell culture. After 14h hours of transfection, the medium was changed and 72 hours post-transfection, co-expression was detected by western blot. As controls, cells expressing pGFP (Invitrogen) plasmid and cells only with culture medium were used. Some variables were tested during the several experiments, such as (i) the time of transfection, which initially was 4h and then prolonged for 14h; and (ii) the time of post-transfection tested (48h, 72h and 96h).

2.9.2.2. ASSAYS WITH THE TESTED COMPOUNDS

The HEK293T and COS-7 cells were seeded in 12-well plates and transfected with a total amount of 750 µg DNA (pEF-DEST51-G46S+STOP) per $1x10^5$ cells using PEI as transfectant agent in a 1:6 ratio. As described in 2.9.1.1, PEI, pDNA and cell medium base was mixed and incubated for 30 min, at RT, before being added to cell culture. After 14h of transfection, medium was supplemented with 50 µM (final concentration) of compound C2, C6 and C18 in 1% DMSO, separately. A reinforcement of the same dose was added 48h after transfection. A parallel negative control with only 1% DMSO was included, as well as, cells expressing pGFP (Invitrogen) plasmid and cells only in culture medium.

2.10. CO-IMMUNOPRECIPITATION ASSAYS

For the co-immunoprecipitation assays (Co-IP), cells were harvested with TripLE Express (Dissociation reagent, Life Technologies), pelleted and lysed by three freeze–thaw cycles in 50 µL of a low salt lysis buffer (50 mM Tris-HCl, pH 8.0, 1% NP-40) containing protease inhibitors (200 µM PMSF). The cell debris was removed by centrifugation at 12800 xg, during 10 min, at 4 °C, and the supernatant was saved. An aliquot of 25 µL (whole cell lysate) was stored for further SDS-PAGEl analysis and the remaining 25 µL was used for Co-IP. For this, 100 µL (1 mg at 10 mg/mL) of SureBeads (BioRad) was washed 3x with 1 mL PBS-T (PBS,

0.1% Tween 20). A sample corresponding to 2.5 µg (1 mg/mL) of the anti-PAH mouse antibody (MAB5278, Millipore) was diluted in 200 µL of Tris-HCl 50 mM, pH 8.0 and incubated with the prepared magnetic beads for 10 min, at RT, with continuous stirring. The SureBeads were magnetized and washed 3x with 1 mL of PBS-T. The supernatant was discarded. The sample was added to the magnetic beads and incubated, with continuous stirring, for 1h, at RT. The Surebeads were again magnetized and washed as in the previous step. After spin down the tubes, to discard residual buffer, the sample was eluted by adding to the magnetic beads 25 µL of 1x Laemmli buffers and incubation for 10 min, at 70ºC. The Surebeads were further magnetized and the eluted sample analysed by SDS-PAGE followed by western blot.

2.11. IMMUNOCYTOCHEMISTRY ASSAYS

For the immunocytochemistry assays, 48h post-transfection, HEK293T cells were rinsed twice with PBS containing 20mM glycine, and fixed for 30 min in a 4% (v/v) paraformaldehyde (Merck). After three washes with PBS, cells were permeabilized for 5 min with 0.1 % (v/v) Triton X-100 (Sigma-Aldrich) in PBS, washed two more times with PBS for 5 min and blocked with 1% (w/v) BSA in PBS for 30 min. After that, cells were incubated for 1 h with the anti-PAH mouse antibody (diluted 1:1000 in 1% (w/v) BSA in PBS). Cells were then washed three times with PBS for 5 min and incubated for 1 h with the FITC-conjugated anti-mouse IgG (GenScript) diluted 1:500 and washed three times again.

Cell slides were mounted in ProLong® Gold antifade reagent with DAPI (Invitrogen) and their immunofluorescence was observed and recorded on an Axiovert 40CFL fluorescence microscope (Carl Zeiss) equipped with an Axiocam MRc5 (Carl Zeiss) camera. Images were processed with the software AxioVision Rel. 4.6.3 (Carl Zeiss).

The same procedure was performed for HepG2 cells in order to visualize the cellular uptake of NPs, with an additional step for actin staining with Rhodamine phalloidin. After cell fixation, cells were permeabilized with 0,1% Triton X100 for 4 minutes and then cells were rinsed three times with 10mM PBS containing 20mM glycine at pH 7.4. The 6.6 mM phalloidin-TRITC (Life Technologies) solution in 10 mM PBS was added to the cells for 30 minutes at room temperature. After cells rinsed three times with 10 mM PBS containing 2 0mM glycine at pH 7.4 and air dried, cell slides were mounted and observed as previously described.

2.12. WESTERN BLOT ANALYSIS

After electrophoretic separation the gel was equilibrated in Towbin buffer (25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3) for 15 min (168). For the transference process, a polyvinylidene difluoride (PVDF) membrane (Amersham Hybond-P;GE Healthcare), previously equilibrated according to manufacture instructions, and the semidry V20 transfer

system (Sci-Plas) were used. The transference was monitored through visualization of the NZYcolour protein marker II used as a control. After transfer the membrane was washed 3x with TBS buffer (140 mM NaCl, 20 mM Tris, pH 7.4), containing 0.1% Tween-20 (TBS-T), for 5 min. To avoid unspecific binding, the membrane was incubated with blocking buffer (5% non-fat milk in TBS-T) for 3 h, at RT. Next, the membrane was washed 3x, 5 min each, with TBS-T and then incubated ON at 4ºC with primary antibody diluted in blocking buffer. The membrane was washed as in the previous step and then incubated with the secondary antibody, diluted in blocking buffer, during 3h at RT. After incubation, the membrane was once again washed 3x, for 5 min each, with TBS-T buffer.

Blots were visualized with ECL Prime Western (GE Healthcare) at a ratio of 0.1 mL/cm^2 of membrane and chemiluminescence was monitored on a ChemiDoc MP Imaging system (BioRad).

For protein detection in NPs assays, the mouse monoclonal Anti-6His tag® antibody (Abcam) diluted 1:500 was used as the primary antibody and the anti-mouse IgG-HRP (W402B, Promega) was used as secondary antibody in a dilution of 1:2500.

In the Co-IP assays the rabbit polyclonal Anti-6X His tag® antibody (Abcam) was used as the primary antibody and the goat anti-rabbit IgG-HRP antibody (BioRad) was used as the secondary antibody. Both antibodies were diluted at 1:1000.

2.13. *PAH* ACTIVITY ASSAY

For hPAHwt activity measurement within NPs, three conditions were established: (i) NPs in homogenized cells; (ii) NPs in SEC buffer; and (iii) NPs in culture medium. As control, the same conditions but with free hPAHwt and a hPAHwt solution at 4ºC were used. For the first condition HepG2 cells were homogeneized and then incubated with the NP and naked hPAHwt, separately, in a 96-well plate, at 37ºC, with shaking for 21h.

After 1h, 2h, 3h, 4h 6h and 21h of incubation, the activity of naked and encapsulated hPAHwt was measured essentially as described by Gersting and collaborators (105) in the activated form (pre-incubation with L-Phe) using the natural fluorescence of L-Tyr and the microplate reader FLUOStar Omega equipped with an injector and a temperature controller. Briefly, the reaction mixture, performed in a 200 mL final volume (96 well microplate), contained 1 mM L-Phe, 0.1 M Na-Hepes, pH 7.0, 1080 U.mL-1 catalase, 5 mg of recombinant 6xHis-hPAHwt (naked or nanoencapsulated) and 5 mM dithiothreitol DTT. After 4 min of pre-incubation, 100 µM ferrous ammonium sulphate was added to the mixture. After 2 min incubation, the reaction was started by the addition of 75 mM BH4. The L-Tyr production was continuously measured using fluorimetric detection (λexc= 260 nm and λem= 310 nm) and quantified using an adequate L-Tyr calibration curve (0.5 – 100 mM). The enzymatic reaction

was performed at a constant temperature of 25ºC and data acquired each 30 seconds during 15 minutes.

CHAPTER IV • **RESULTS AND DISCUSSION**

1. EFFECT OF 3HQS ON p.G46S AGGREGATION

1.1. *IN VITRO* STUDIES

1.1.1. EXPRESSION AND PURIFICATION OF MBP-hPAH-G46S

For the *in vitro* studies of the p.G46S variant, the recombinant protein MBP-hPAH-G46S was produced in *E. coli*, at 28ºC, during 5 h, upon induction with 1 mM IPTG.

In order to obtain a sample with a high purity degree, two chromatographic steps were used. The first purification procedure involved the use of affinity chromatography in order to purify the MBP-recombinant protein from the remaining proteins present in the soluble fraction using an amylose resin. The MBP tag present in the recombinant protein has a high affinity to the amylose resin, and was further eluted using 10 mM maltose in the chromatographic eluent. The amylose purification profile is presented in Figure IV.1.

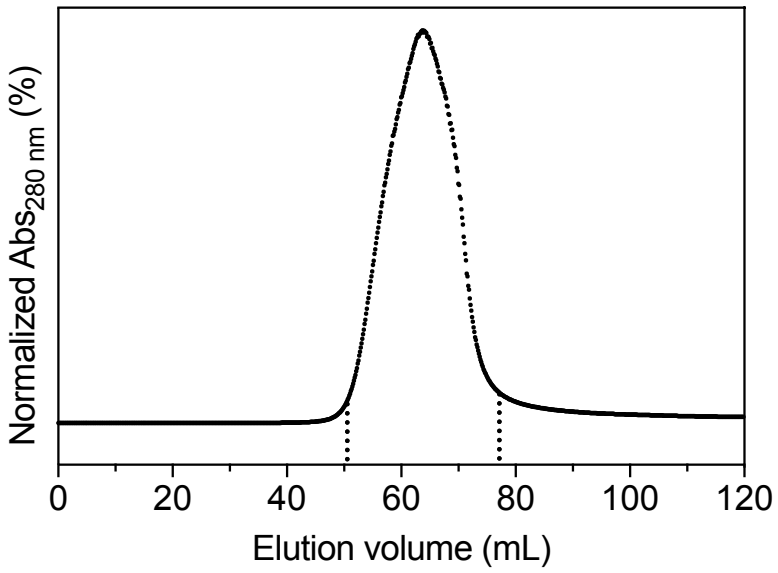


FIGURE IV.1. Chromatographic profile of recombinant MBP-hPAH-G46S, obtained by affinity chromatography using an amylose resin. The peak corresponds to the elution of the MBP recombinant protein (51-77 mL). The dashed lines indicate the fractions collected and pooled for further purification by SEC.

Upon purification of the MBP recombinant protein (fractions corresponding to the dashed lines in Figure IV.1), a second procedure using SEC was used to isolate the biological relevant tetramers The SEC chromatogram (presented in Figure IV.2) shows the obtained oligomeric profile.

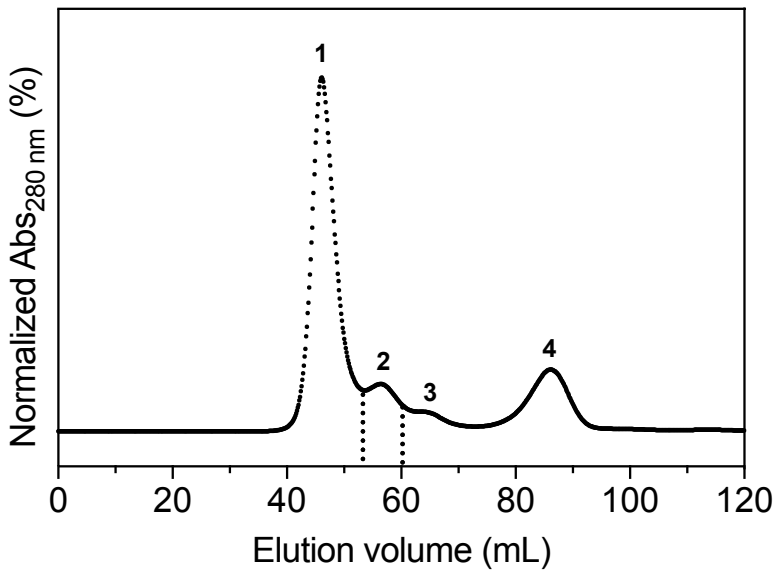


FIGURE IV.2. Chromatographic profile of recombinant MBP-hPAH-G46S variant, obtained by size exclusion chromatography (SEC). Peak 1 correspond to aggregates and higher-order oligomeric forms (42-52 mL), peak 2 represents the tetrameric form (55-60 mL), peak 3 the dimeric form (62-65 mL) and peak 4 the maltose binding protein (MBP) (82-90 mL). The dashed lines indicate the fractions that were collected and utilized for further studies.

The utilized chromatographic separation system allowed the isolation of different oligomeric forms of MBP-hPAHG46S, such as high-order oligomeric forms eluted in the column void volume (Figure IV.2, peak 1), the tetrameric forms (≈ 262 kDa; Figure IV.2, peak 2), dimeric forms (≈ 194 kDa; Figure IV.2, peak 3), and MBP (≈ 42 kDa; Figure IV.2, peak 4). As shown in figure IV.2, the MBP-hPAH-G46S variant was mainly expressed as soluble aggregates and only a small amount was present as. Interestingly, using the described expression as chromatographic conditions, monomeric forms of MBP-hPAH-G46S were not observed. After SEC, the fractions corresponding to the tetramers were collected (indicated by dashed lines) and concentrated until reach 7-9 mg/mL of protein concentration.

As control of the purification procedures and protein integrity, samples of the isolated proteins collected from the amylose resin (Figure IV.3, line 1) and after SEC (Figure IV.3, line 2) were analysed through SDS-PAGE (Figure IV.3).

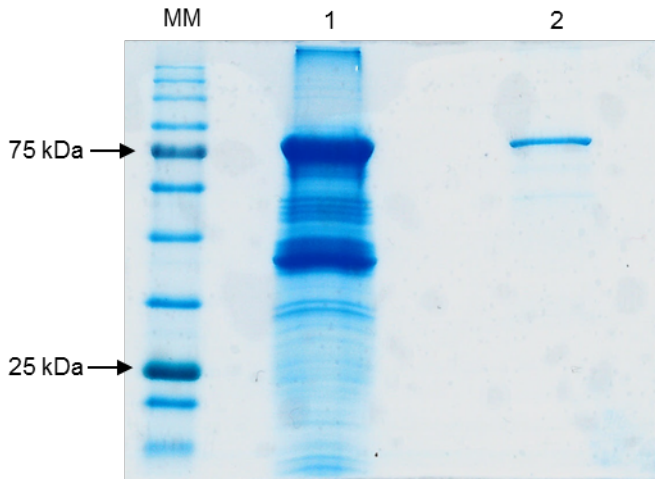


FIGURE IV.3. SDS-PAGE analysis of amylose and SEC purification of MBP-hPAH-G46S. (1) Sample from collected and pooled fractions from amylose chromatography (dashed line in Figure V.1), showing several proteins including MBP (≈46 kDa); (2) Sample from collected and pooled fractions after SEC (dashed lines in Figure V.2) showing a main band corresponding to the MBP-hPAH-G46S (≈ 96 kDa). (MM) Protein molecular mass marker (NzyColour protein marker II; Nyztech).

1.1.2. EVALUATION OF FXA INHIBITION BY TESTED COMPOUNDS

The library of 3HQs compounds synthesized by the Bioorganic group at iMed.ULisboa, were already tested for their ability to modulate the activity and stability of the hPAHwt (Raquel Lopes master thesis, under conclusion). As the above compounds demonstrated affinity for the hPAHwt and for some of them a stabilizing effect was also observed, we hypothesized that they might be capable of inhibiting hPAH-G46S aggregation *in vitro*.

However, before proceed with G46S aggregation assays, an enzymatic assay in the presence of the compounds and a synthetic FXa substrate was performed in order to rule out any direct effect of the 3HQs derivatives upon the FXa activity. As FXa substrate the N-carbobenzyloxy-glicyl-glicyl-arginyl-7-amino-4-methyl-coumarin (N-CBZ-Gly-Gly-Arg-AMC; I-1140; Bachem) was used. The protease FXa cleaves after the arginine (Arg) residue of the sequence Ile-Glu/Asp-Gly-Arg releasing the AMC moiety of the substrate which presents fluorescence (λ_{exc} 360 nm; λ_{em} 460 nm) which was measured along time. The obtained curves allowed calculating the enzymatic activity of FXa and concomitantly to infer for FXa inhibition. The lower the inhibition of FXa by the tested 3HQs compounds, the greater the fluorescence intensity detected, meaning that the compounds could be used to test for their effect on the aggregation of hPAH-G46S after MBP release by FXa. In Figure IV.4 is exemplified the obtained fluorescence in the presence of compounds with low, medium and high level of FXa inhibition.

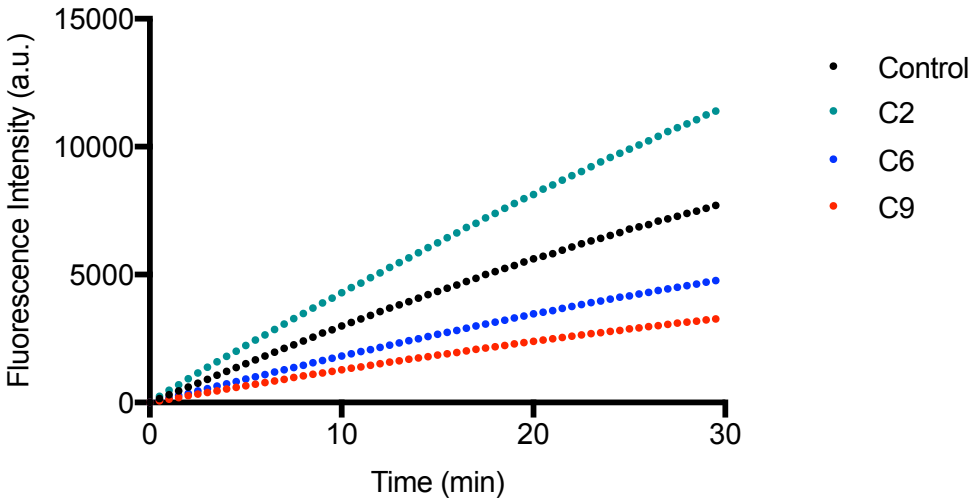


FIGURE IV.4. Enzymatic assay to monitor the inhibition of FXa activity by the tested 3HQs derivatives. The figure shows three examples of assays performed in the absence (•) and presence of compounds showing different behaviour namely C2 (•) a non-inhibitor, C6 (•) mild inhibitor and C9 (•) a strong inhibitor.

The FXa inhibition studies (presented in Table IV.1) showed that the majority of the tested compounds did not inhibit FXa activity. Only a slight degree of inhibition was found for C6 (18% of inhibition) and C8 (15% of inhibition), while a more pronounced effect was detected for C4 (50% of inhibition) and C9 (73% of inhibition).

TABLE IV.1 – Factor Xa activity in the absence and presence of tested 3HQ compounds at 100 μM in two independent assays (each performed in triplicate).

	Assay 1		Assay 2	
	Activity ± SD ($AU.min^{-1}$)	Inhibition (%)[a]	Activity ± SD ($AU.min^{-1}$)	Inhibition (%)[a]
Control (1% DMSO)	296	0	367	0
C2	426 ± 85.6	0	-	-
C4	147 ± 16.3	50	-	-
C6	242 ± 30.0	18	-	-
C8	253 ± 2.3	15	-	-
C9	81 ± 7.3	73	-	-
C10	-	-	365 ± 53.8	1
C11	-	-	628 ± 19.0	0
C12	-	-	646 ± 38.8	0
C14	-	-	660 ± 35.3	0
C15	-	-	598 ± 64.4	0
C16	-	-	697 ± 32.1	0
C17	-	-	374 ± 20.8	0
C18	-	-	718 ± 44.4	0
C19	-	-	696 ± 20.4	0
C20	-	-	694 ± 25.4	0
C21	-	-	668 ± 11.8	0
C22	-	-	537 ± 54.4	0

Notes: (SD) standard deviation. Activity values were determined as initial velocity (V_0). [a] FXa protease inhibition in percentage was calculated by considered the activity in 1% DMSO and in the absence of compound as 100%.

According to the obtained data only C4 and C9 should be excluded to proceed with the hPAG-G46S aggregation assays and care should be taken when analysing the results obtained for C6 and C8.

1.1.3. AGGREGATION ASSAYS

As mentioned before, the MBP-hPAH-G46S provides an excellent system to evaluate the aggregation of the p.G46S variant as when the MBP fusion partner is removed, the hPAH-G46S protein aggregates as non-amyloid like fibrils (169). In the hPAH-G46S aggregation studies two different control assays were performed (Figure IV.5): (i) a positive control reaction resulting from the MBP-hPAH-G46S cleavage by FXa in the presence of 1% DMSO and in the absence of compounds and; (ii) a negative control reaction including MBP-hPAH-G46S in the absence of FXa and compounds. For all the assays, aliquots of the aggregation reaction were withdrawn at the end (180 min) of the reaction and were analysed by SDS-PAGE to confirm the extent of MBP cleavage by FXa

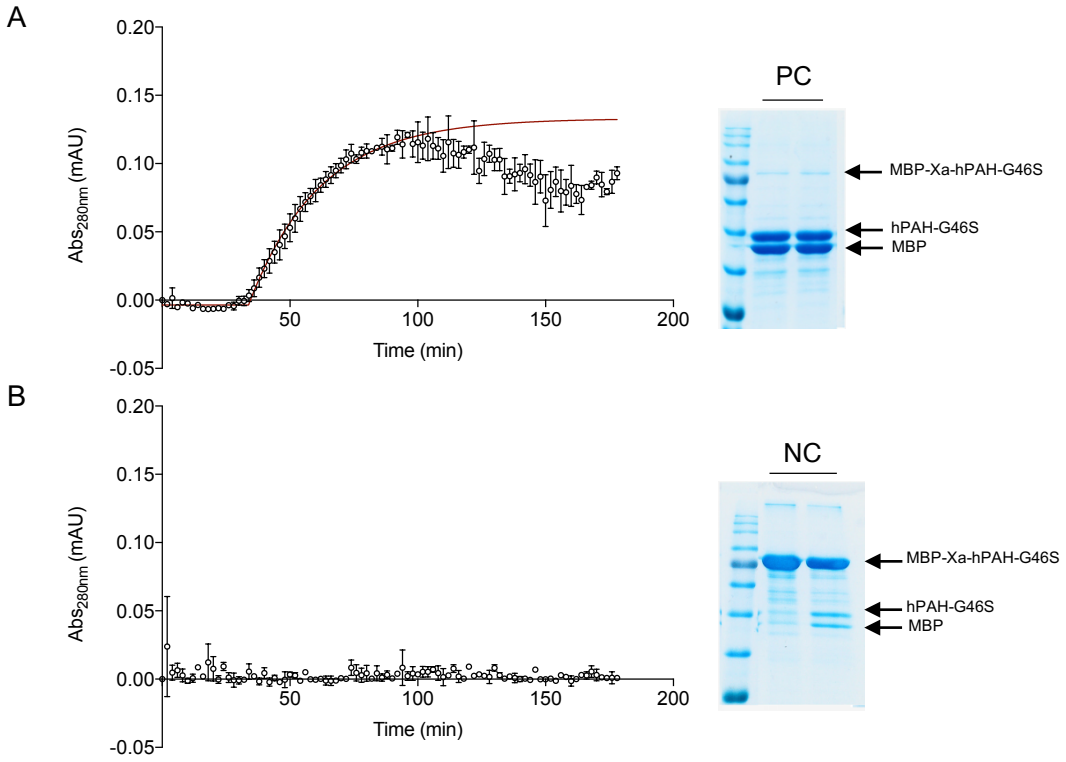


FIGURE IV.5. Control assays of the hPAH-G46S aggregation reaction followed by turbidimetry. (A) Positive control (PC) reaction resulting from the MBP-hPAH-G46S cleavage by FXa in the presence of 1% DMSO and in the absence of compounds. (B) Negative control (NC) reaction including MBP-hPAH-G46S in the absence of FXa and compounds. The SDS-PAGE analysis at time 180 min of reaction is shown for the replicates of the assay. Data represents the mean±SD (error bars).

Although the previous results of the evaluation of FXa inhibition immediately excluded 2 of the 17 tested 3HQ compounds, they were considered in the hPAH-G46S aggregation assays.

However, it was known in advanced that absence of aggregation regarding any of those 2 compounds (C4 and C9) and also C6 and C8, could be due to FXa inactivation and, consequently, to the non-cleavage of the MBP partner which keep the protein stable.

The obtained results are exemplified in Figure V.6, for some of the tested compounds.

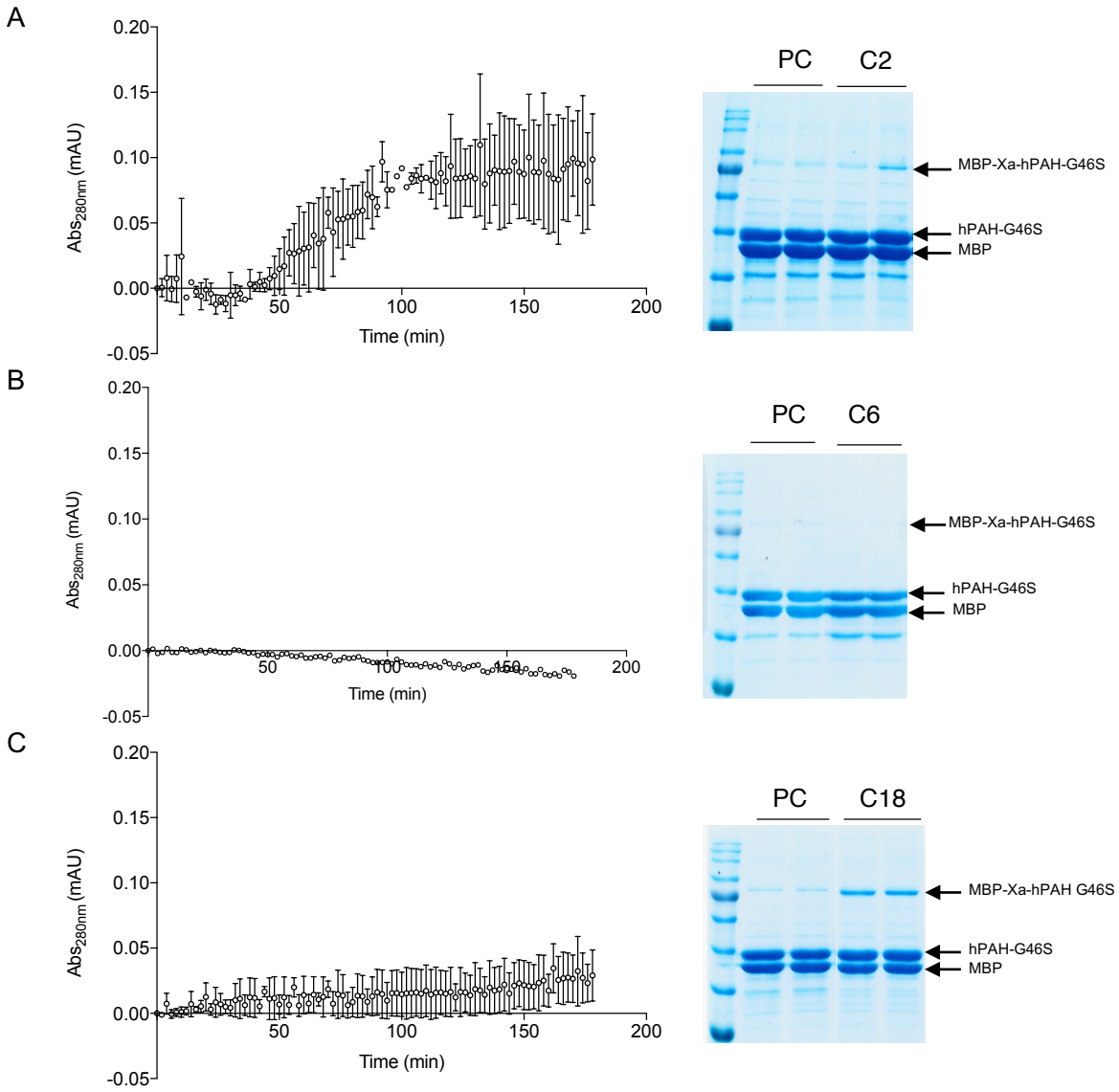


FIGURE IV.6. The hPAH-G46S aggregation reaction followed by turbidimetry in the presence of 3HQs derivatives, namely C2 (A), C6 (B) and C18 (C). (PC) Positive control reaction resulting from the MBP-hPAH-G46S cleavage by FXa in the presence of 1% DMSO and in the absence of compounds. The SDS-PAGE analysis at time 180 min of reaction is shown in the right panel for the replicates of the assay. Data represents the mean±SD (error bars).

When following *in vitro* aggregation of the hPAH-G46S, the analysis of the obtained curves allow us to calculate the time needed to initiate aggregation (t_{lag} or lag phase) as well as the rate of protein aggregation (k). The t_{lag} and k obtained for the 17 compounds tested are shown in Table IV.2.

TABLE IV.2. Aggregation parameters of hPAH-G46S, followed by turbidimetry, in the absence and presence of tested 3HQ compounds at 100 µM.

	Assay 1		Assay 2	
	t_{lag} (min)	k (AU.min^{-1})	t_{lag} phase (min)	k (AU.min^{-1})
Positive Control (1% DMSO)	35	0.040	60	0.019
Negative Control	∞	-	∞	-
C2	43	0.098	-	-
C4	∞	0	-	-
C6	∞	0	-	-
C8	∞	0	-	-
C9	41	0.046	-	-
C10	∞	0	-	-
C11	-	-	∞	0
C12	-	-	106	$6.5x10^{-6}$
C14	-	-	55	0.021
C15	-	-	104	$2.4x10^{-5}$
C16	-	-	∞	0
C17	-	-	94	0.0080
C18	-	-	∞	0.0013
C19	-	-	∞	0
C20	-	-	ND	ND
C21	-	-	ND	ND
C22	-	-	ND	ND

Notes: (k) aggregation rate, correspond to the slope of each curve; (t_{lag}) lag phase. Both parameters were calculated through a plateau followed by one phase association equation. (ND) Not determined. (AU) arbitrary units.

In the positive control (PC; Figure IV.5.A), the cleavage of MBP-hPAH-G46S fusion protein by FXa leaded to the aggregation of the unstable hPAH-G46S mutant enzyme with a t_{lag} of 35 min (Table IV.2), while in the negative control (NC; Figure IV.5.B), in the absence of FXa, no increase in absorbance was observed (t_{lag} ∞) meaning that there was no protein aggregation. The hPAH-G46S aggregation profile in presence of C2 (FigureI IV.6.A) is similar to the PC with a t_{lag} of 43 min which means that C2 is a poor aggregation inhibitor. On the contrary, C6 (Figure IV.6.B) and C18 (Figure IV.6.C) are strong and mild aggregation inhibitors, respectively, as the hPAH-G46S aggregation profile has no absorbance increasing with t_{lag} impossible to be calculated (∞; Table IV.2).

As such, according to the obtained t_{lag} and k (shown in Table IV.2) from the 17 compounds tested, we were able to group them in three different categories according to the obtained aggregation parameters, namely strong (C6, C8, C10, C16, C19); mild (C18, C12, C15, C17); and poor (C2, C9, C14) inhibitors. Regarding C4 and C9, conclusions cannot be stablished as the compounds showed an inhibitory activity against FXa.

1.2. *IN CELLULO* STUDIES

For the *in cellulo* studies of hPAH-G46S aggregation, C2, C6 and C18 were the selected compounds, as they showed, *in vitro*, three different outstand behaviors. These assays were performed in HEK293T cells once they do not express the hPAH protein constitutively.

A preliminary assay was performed in order to optimize the transfection conditions and the immunocytochemistry procedure. In these assays the HEK293T cells were transfected with different amounts of the pG46S expression construct namely, 143 ng, 285 ng and; 570 ng. Non-transfected cells were used as control.

As shown in figure IV.7, the HEK293T cells were successfully transfected with the pG46S expression construct and as expected, protein expression was dependent of the amount of the DNA used, being 570 ng the optimal quantity to express the p.G46S variant in HEK293T cells (Figure IV.7.D).

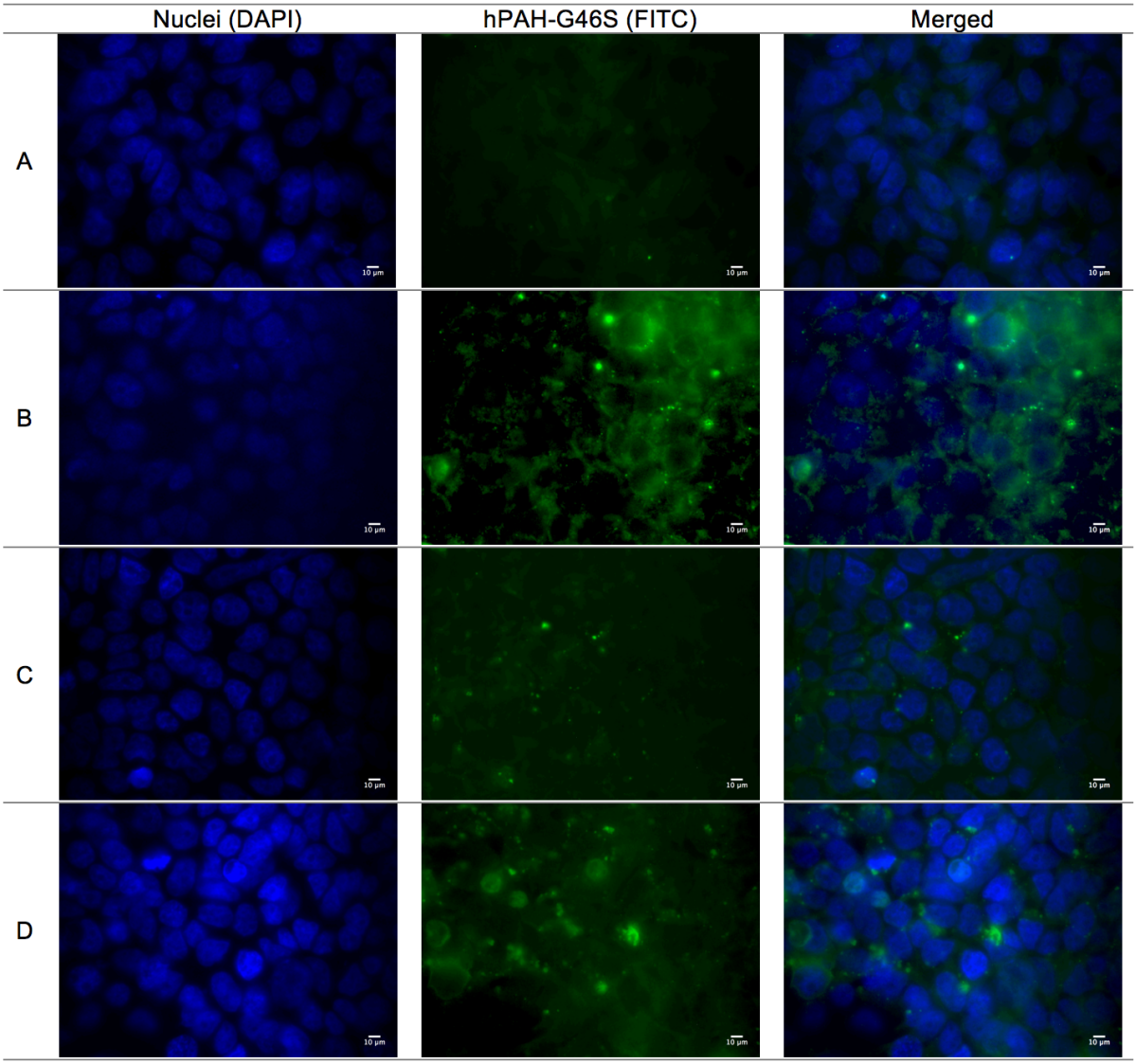


FIGURE IV.7. Transfection efficiency of HEK293T cells with pG46S constructs evaluated by Immunocytochemistry analysis. Cells were transfected using different amounts of plasmid pG46S: (B) 142 ng; (C) 286 ng and (D) 572 ng. Non-transfected cells were used as control (A).

Interestingly, while performing the preliminary optimization of the immunocytochemistry assay, it was possible to observe that the hPAH-G46S when expressed in mammalian cells, such as HEK293T, seemed to be aggregated as their expression pattern is not homogenous but appeared as intracellular fluorescent dots. These data corroborated the data obtained *in vitro* (MBP-hPAH-G46Saggregation assays) indicating that also in mammalian cells the protein tends to aggregate.

This was definitely a good point start that led us to evaluate *in cellulo* the modulation capacity of C2, C6 and C18 compounds regarding the hPAH-G46S aggregation profile. For the immunocytochemistry assay, the hPAH-G46S mutant protein (positive control) and the hPAHwt protein (negative control) were expressed, separately, in HEK293T cells using the

previous optimized conditions. Cells expressing hPAH-G46S were incubated with 50 μM of each compound in 1% DMSO and also with 1% DMSO, as a blank control.

As shown in Figure IV.8.1 and Figure IV.8.2, HEK293T were successfully transfected as they expressed either the hPAHwt (Figure IV.8.1.C) or hPAH-G46S (Figure IV.8.1D and Figure IV.8.2 E to H).

Analysis of the immunocytochemistry assays presented in Figure IV.8.1 clearly showed that the hPAHwt and hPAH-G46S proteins have different patterns of cell expression. In fact, the hPAHwt it is uniformly expressed within HEK293T cells (Figure IV.8.1.C) while the p.G46S variant shows an uneven pattern of expression observed by intracellular fluorescent dots (Figure IV.8.1.D), suggesting protein aggregation.

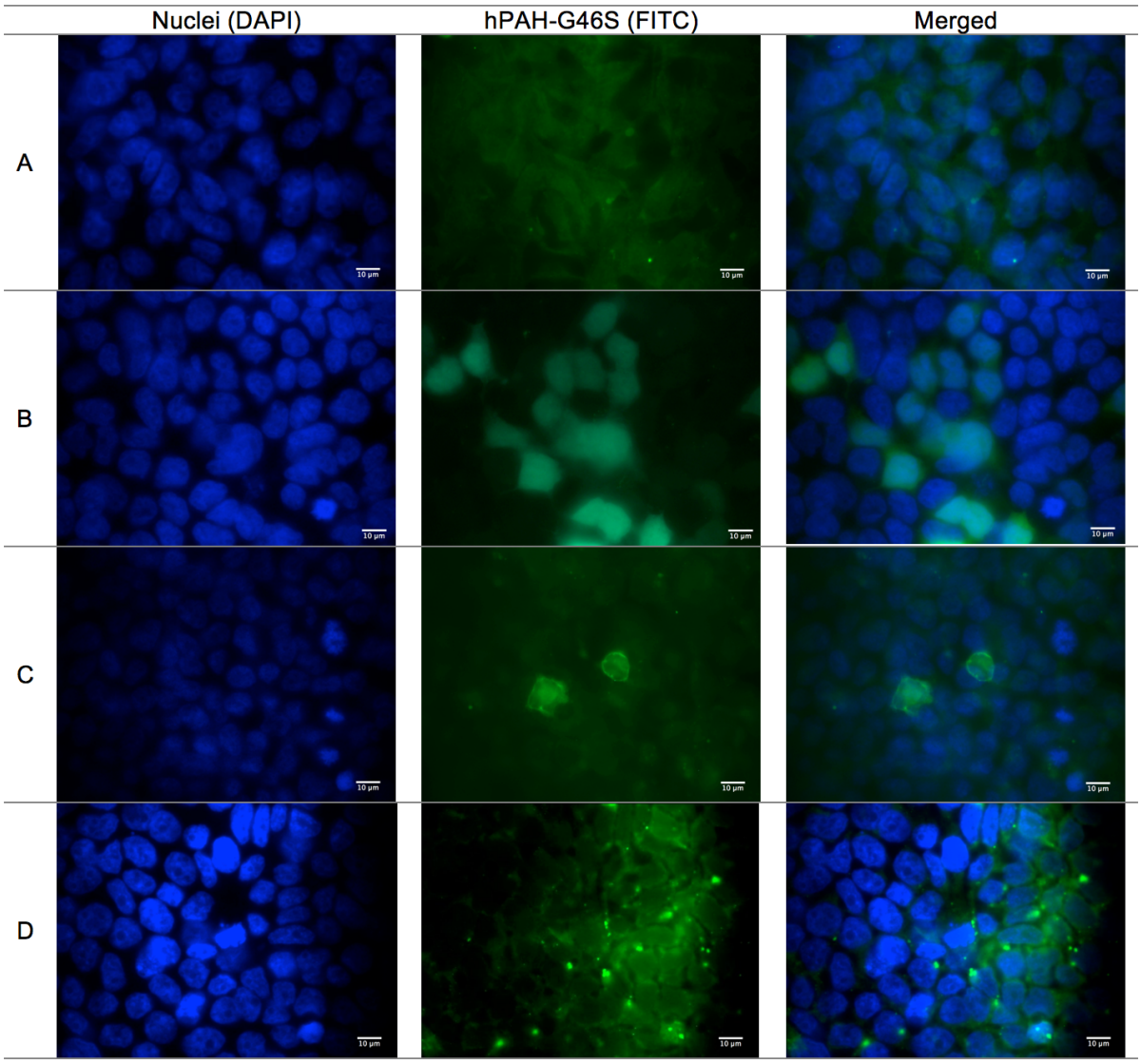


FIGURE IV.8.1. *In cellulo* evaluation of hPAH-G46S aggregation profile in HEK293T cells, after incubation with 50 μM of 3HQs derivatives in 1% DMSO. The assay controls included control cells (A), GFP protein expression (B), hPAHwt protein expression (C) and hPAH-G46S protein expression (D) cells incubated with 1% DMSO (E). The green dots indicate protein aggregation.

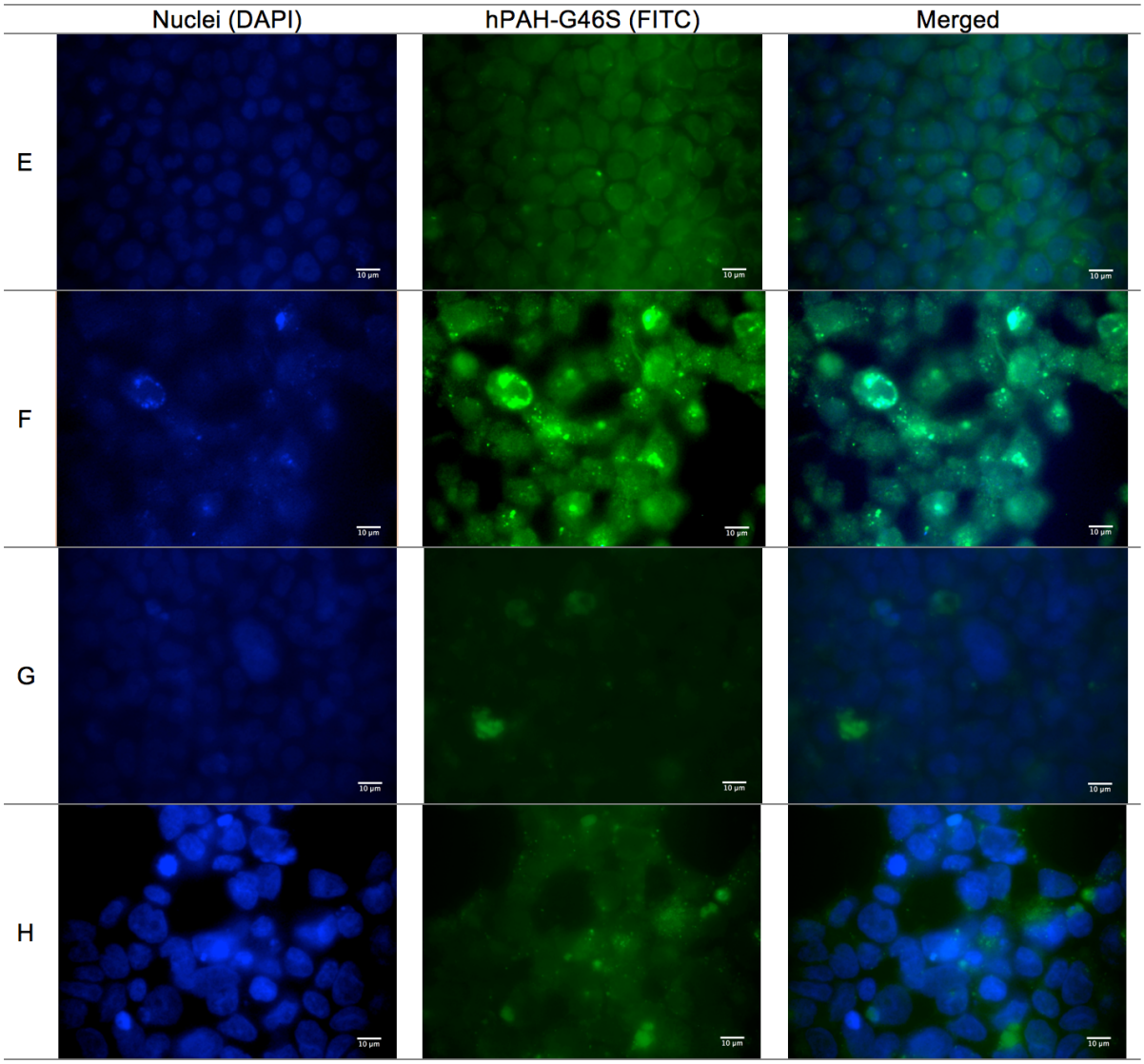


FIGURE IV.8.2. *In cellulo* evaluation of hPAH-G46S aggregation profile in HEK293T cells, after incubation with 50 μM of 3HQs derivatives in 1% DMSO. Cells incubated with 1% DMSO (E), C2 (F), C6 (G) and C18 (H).

Regarding the hPAH-G46S expression in presence of the tested compounds, and as expected from the *in vitro* aggregation assays, only with C6 (Figure IV.8.2.G) and C18 (Figure IV.8.2.H) a reversion of the expression pattern of p.G46S is altered with no visible intracellular fluorescent dots, suggesting that protein aggregation was reverted by these 3HQs derivatives. However, when observing the expression pattern of hPAH-G46S in presence of C2 (Figure IV.8.2.F) the intracellular fluorescent dots are still present corroborating the *in vitro* aggregation assay. In fact in this assay the C2 3HQ derivative was unable to inhibit aggregation of the hPAH-G46S (Figure IV.8.2.F) Interestingly, cells expressing the hPAH-G46S and incubated in the presence of 1% DMSO, although presenting an image

characteristic of aggregated protein, the fluorescent green dots were less intense than expected (Figure IV.8.2.E).

2. STUDIES OF hPAH-G46S AND MOLECULAR CHAPERONE INTERACTIONS

2.1. OPTIMIZATION ASSAYS

It is known from previous expression studies in eukaryotic cell lines, that the immunoreactive level of the p.G46S variant is very low (~3%). It has been hypothesized that the variant protein will be rapidly degraded by the cell protein quality control system, thus leading to low cellular steady-state levels. As the mechanism by which this degradation occurs is unknown, the aim of this experiment was to co-express, in eukaryotic cell lines, the p.G46S variant protein and key molecular chaperones, which, according to literature, might be involved in the proteostasis network that handles the p.G46S hPAH variant. Following co-expression, a Co-IP technique will allow inferring for potential interactions among the p.G46S and target molecular chaperones.

The antibodies used for Co-IP took advantage of the presence of a 6xHis tag in the expressed molecular chaperones and first several assays were performed in order to optimize this experimental approach namely: (i) different eukaryotic cell lines; (ii) transfection reagent; (iii) time of contact between cells and transfection reagents and; (iv) incubation time.

Regarding the cell lines, throughout the process two different cell lines were used, HEK293T cells (as they do not constitutively express PAH) and COS-7 cells (as they have been used in several hPAH expression studies). The cells were transfected with the DNA constructs expressing the p.G46S and molecular chaperones either isolated or simultaneously (co-transfection).

First we aimed to confirm cell expression of both proteins, separately or together. When using COS cells, by increasing contact time between cells and transfection reagents from 4 to 14 h, and rising the incubation time from 48 to 72 h, it was possible to obtain a slight signal of the p.G46S protein (Figure IV.9.I), by western blot analysis, but no signal of molecular chaperone (Figure IV.9.II).

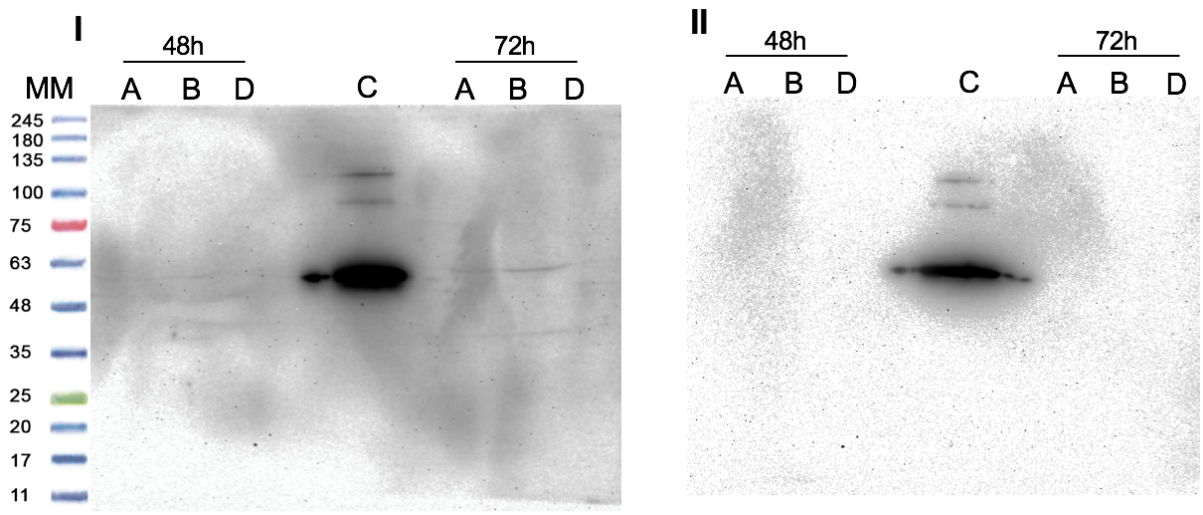


FIGURE IV.9. Western blot analysis of hPAH-G46S (I) and the molecular chaperone 6xHis-HSPAB1 (II) expressed in COS-7 cells. Cells were transfected using PEI, incubated for 48 h and 72 h and different cellular fractions were analyzed: total lysate (A), supernatant (B) and pellet (D). The 6xHis-hPAHwt was used as control (C). The anti-hPAH and anti 6xHis primary antibodies were used in I and II respectively.

Facing these results we hypothesized that the obtained data could be a result of poor transfection efficiency. So, in an attempt to increase transfection efficiency and thus protein expression, the transfection reagent was changed from PEI to lipofectamine®. This assay was performed in HEK293T and COS-7 cells, maintaining the 72 h of incubation. However, the use of lipofectamine® did not significantly improve protein expression (data not shown), so the use of PEI was maintained for economic reasons.

As the result of the optimization assays a contact time between cells and transfection reagents of 14 h and an incubation time for protein expression of 72 h was chosen for co-transfection (Figure IV.10). The same results were obtained with both HEK293T and COS-7 cells (results not shown), so either of this cell lines can be used in this assay.

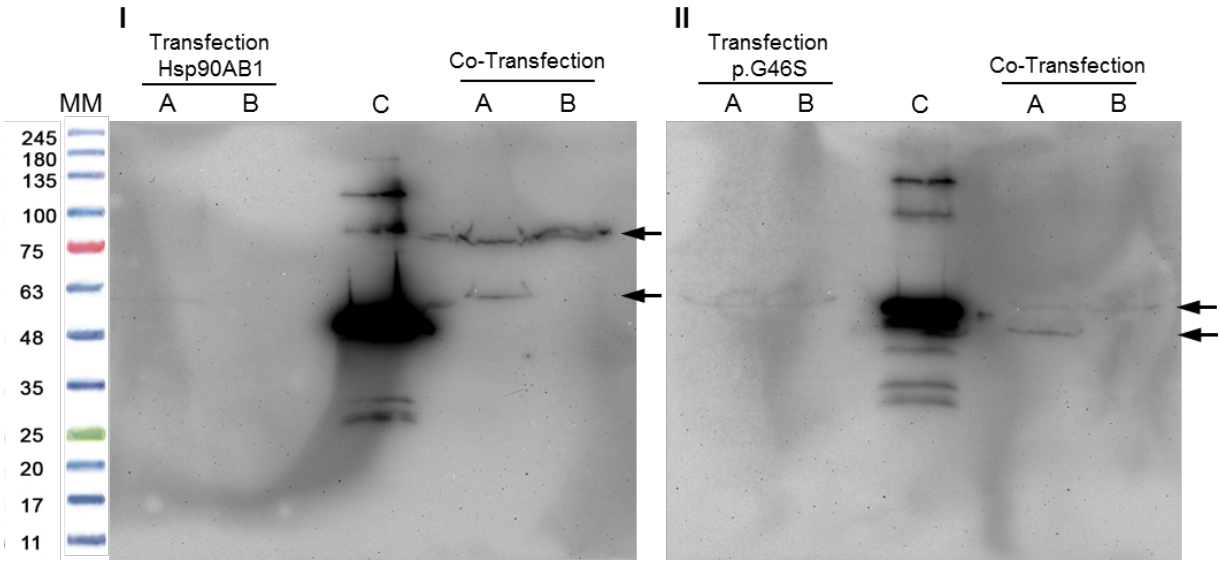


FIGURE IV.10 – Western blot analysis of the molecular chaperone 6xHis-Hsp90AB1 (I) and hPAH-G46S (II) in transfected and co-transfected COS-7 cells after 72h incubation. The total cell lysate (A) and the supernatant fraction were analysed. The 6xHis-hPAHwt was used as control (C). Immunoblot was done against Hsp90AB1 (1) and G46S (2). The anti 6xHis and anti-hPAH primary antibodies were used in I and II respectively. The band corresponding to the molecular chaperone (I) and the p.G46S (II) in the co-transfection assay are indicated by an arrow.

Interestingly, the band corresponding to the Hsp90AB1 (≈ 90 kDa; Table IV.1) was only observed in the co-transfection assay (Figure IV.10.I).

Considering the obtained data the study of hPAH-G46S/molecular chaperones interactions through Co-IP was then initiated.

2.2. CO-IMMUNOPRECIPITATION ASSAYS

The molecular chaperones firstly selected for testing were chosen based on their described main role (Table III.1) and included three members of the HSP70 family (HspA8; HspA2 and HspH1) and one member of the HSP90 family (Hsp90AB1). The SQTM1 protein, a member of the sequestosome-1, was also selected as it has been described as being involved in the degradation pathway of polyubiquitin-containing bodies.

Hence, HEK293T cells were co-transfected with the DNA constructs expressing the p.G46S variant and one of the molecular chaperones, and incubated for 72 h. Preliminary data from the CoIP experiments (Figure IV.11) shown unspecific signals for each of the molecular chaperones tested with no clear band with the expected MM (indicated by an arrow in Figure IV.11). However, each of the analysed Co-IP seemed to present a different pattern which may indicate some differences in the hPAH-G46S/molecular chaperone interactions. However, further assays are needed to elucidate the above mentioned phenomenon.

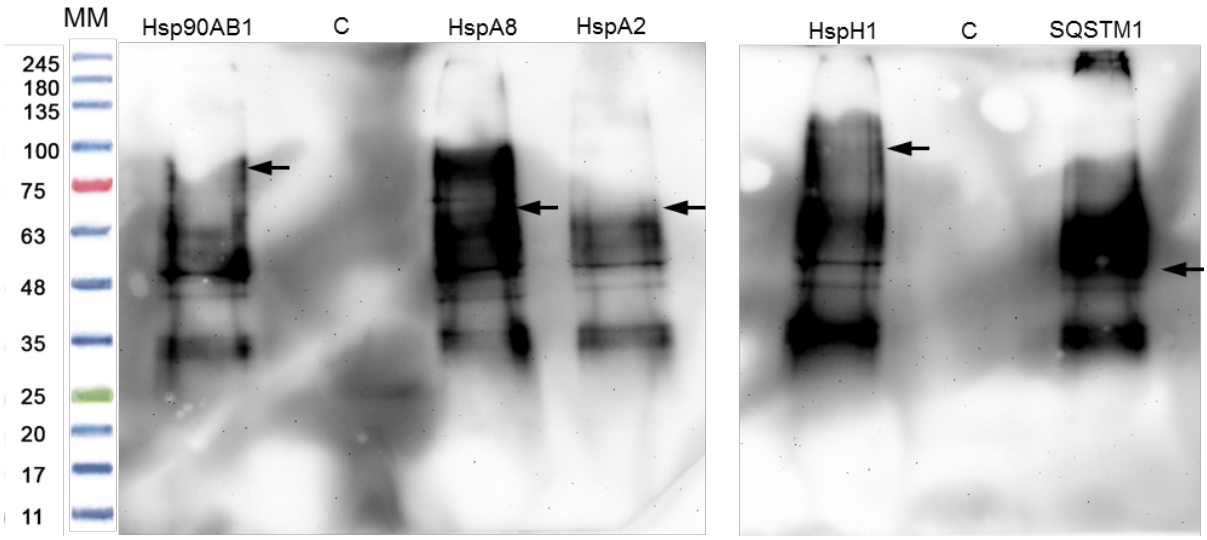


FIGURE IV.11 – Western blot analysis of hPAH-G46S/molecular chaperones interactions using Co-IP upon co-expression in HEK293T cells. The molecular chaperones tested included Hsp90AB1, HspA8, HspA2, HspH1 and SQSTM1. The anti-hPAH antibody was used in the immunoprecipitation step and for the western blot analysis an anti- 6xHis was used as the primary antibody.

3. *IN CELLULO* EVALUATION OF hPAHwt LOADED NANOPARTICLES

When formulating a protein aiming development of ERT an optimized balance between functionality and the gain of thermodynamic stability should always be sought to attain the best protein fitness possible. One of the main strategies applied to pharmaceutical protein stabilization, include their immobilization into biodegradable carriers, such as CS NP.

Following the successful incorporation of hPAHwt in CS NP with the *in vitro* maintenance of the enzymatic activity, L-Phe activation, thermostability and protein conformation, our research group now aimed to assess the NP cellular uptake, as well as, to evaluate the activity of hPAHwt loaded CS NP in the cellular fractions. This will allow us to stablish if the CS NP are able to stabilize the enzyme *in cellulo* and provide a potential safe and effective nano drug delivery system (nano-DDS).

3.1. EXPRESSION AND PURIFICATION OF hPAHwt

The recombinant protein hPAHwt was produced in *E. coli*, at 37ºC during 4 h of induction with 1 mM IPTG. Under these conditions, the overexpressed recombinant protein was mostly recovered in the soluble fraction (results not shown).

In order to have a sample with high purity grade, which is necessary to the hPAHwt assays, two chromatographic procedures were used namely an IMAC using a Ni-NTA resin and SEC to isolate the biological relevant tetrameric form.

The IMAC purification profile of hPAHwt is represented in figure IV.12. The soluble fraction of the lysate was washed with increasing concentrations of imidazole solutions in order to purify the protein of interest, As observed in Figure IV.12 (line 2 to 6) the bacterial proteins present in the soluble fraction were eluted during the washing steps.

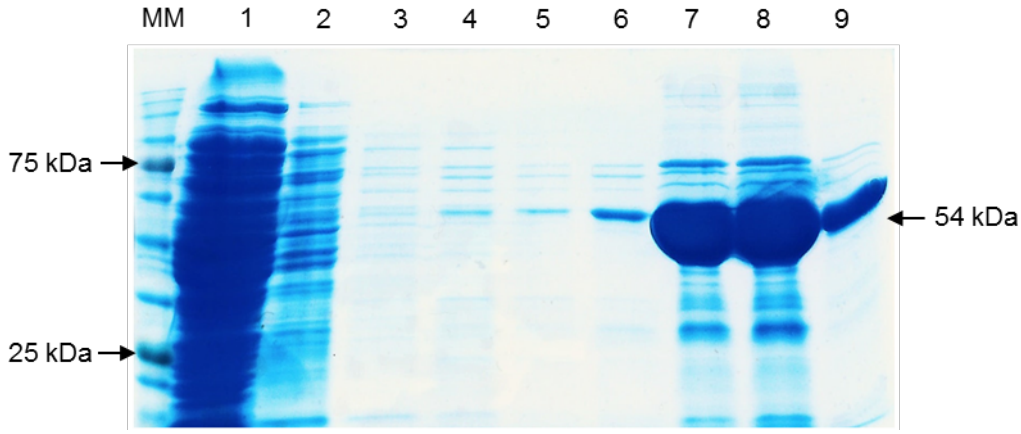


FIGURE IV.12 – SDS-PAGE analysis of IMAC purification profile of hPAHwt. (1) Flow-through; Washing steps using 6xHis lysis buffer containing 20 mM imidazole (2 and 3),50 mM imidazole (4 and 5) and 75 mM imidazole (6). The recombinant 6xHis-hPAHwt protein was eluted using 6xHis lysis buffer containing 250 mM imidazole (7 to 9). (MM) Prestained protein molecular weight marker (NzyColour protein marker II, Nyztech). The MM of the recombinant 6xHis hPAHwt is indicated (≈ 54 kDa).

As the IMAC purification is a common procedure in the Met&Gen group lab, the elution scheme (Figure IV.12; lines 7 to 9) had been optimized in previous work. Eluting the protein with 3x500 μL of 250 mM imidazole buffer is the optimum elution scheme to obtain an adequate volume (1.5 mL) to be immediately injected in the SEC column (5 mL loop) without further manipulations. Through SEC, the tetrameric form of the hPAHwt protein was isolated (Figure IV.13, peak 3).

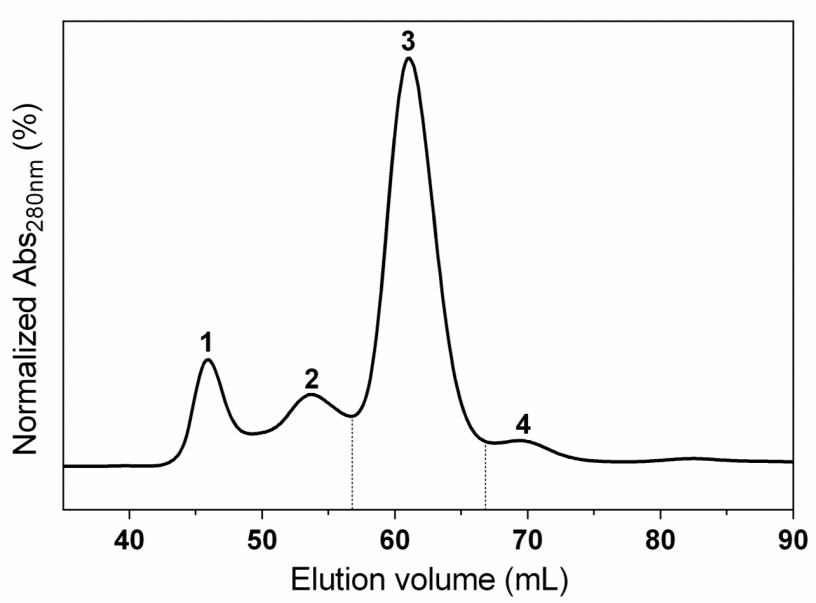


FIGURE IV.13. Chromatographic profile of recombinant hPAHwt, obtained by size exclusion chromatography (SEC). Peak 1 represents higher-order oligomeric forms (44-47 mL), peak 2 represents the octameric form (52-56 mL), peak 3 the tetramer (56-66 mL) and peak 4 the dimer (69-71 mL).

The developed chromatographic separation system allowed the isolation of different oligomeric forms of hPAHwt, such as high-order oligomeric forms eluted in the column void volume (Figure IV.13, peak 1), octamers (≈ 440 kDa; Figure IV.13, peak 2), tetramers (≈ 220 kDa; Figure IV.13, peak 3) and dimers (≈ 110 kDa; Figure IV.13, peak 4). As shown in the chromatogram presented in Figure IV.13, the hPAHwt was mainly purified and tetramers (60.4%), with a small percentage of dimers (6.5%), octamers (22.4%) and aggregates (10.7%). Using these chromatographic conditions, it was not possible to detect any peak corresponding to the monomeric form of hPAHwt. After SEC, the fractions corresponding to the tetramers were collected (Figure IV.13; dashed lines), pooled and concentrated until reach 7-9 mg/mL of protein concentration.

3.2. PROPERTIES OF NANO ENCAPSULATED hPAHwt

After the nanoencapsulation procedure, using the controlled conditions previously optimized (CS:TPP mass ratio of 5:1, initial CS pH 5.5), the system was characterized regarding a hPAHwt loading of 250 µg/mL.

Through the DLS technique, mean size of empty and loaded CS:TPP nanoparticles were determined and are presented in Table IV.3. Mean size was slightly increased in loaded NP without extensive PI destabilization (Table IV.3). In terms of zeta potential either the empty or the loaded CS-NP have a positive value of 25 ± 1 mV and 20 ± 2 mV, respectively, indicating that after protein encapsulation the surface charge remains similar.

Regarding encapsulation efficiency (E.E.), a high incorporation efficiency of 82 ± 2 (% w/w) was observed and was also expected, due to the previous work developed for optimization of formulation conditions.

Table IV.3 – Characteristics of empty and loaded chitosan nanoparticles

	E.E. (% w/w)	Zeta Potential (+mV)	Mean Size (μm)	P.I.
Empty Nanoparticles	N.A.	+25 ± 1	263 ± 3	0.239 ± 0.005
Loaded Nanoparticles[(a)]	82 ± 2	+20 ± 2	290 ± 4	0.186 ± 0.012

Notes: (P.I.) polydispersity index; (E.E.) encapsulation efficiency; (N.A.) Not Applicable. The data represent the mean and SD of three independent assays. [(a)]Loading of 250 μg/mL hPAHwt.

3.3. EVALUATION OF hPAHwt CONTENT AND FUNCTION IN DIFFERENT CELLULAR FRACTIONS

The CS:TPP NP loaded with hPAHwt where incubated with hepatocyte derived HepG2 cells in order to evaluate the NP internalization (cellular uptake) and the protein content in different cellular fractions, namelly: (i) culture medium supernatant; (ii) whole cell lysate; (iii) lysate pellet and; (iv) lysate supernatant.

The aim was to build a cellular model to evaluate if cellular uptake of NPs occurred and concomitantly the cell could gain the capacity to metabolize L-Phe into L-Tyr through the hydroxylation reaction.

The content of the hPAHwt protein loaded in the NP was evaluated in different cellular fractions for different times of incubation, at 37ºC. In Figure IV.14 the western blot analysis of the cellular fractions after 1 h, 6 h and 16 h of incubation with CS-NP-hPAHwt is shown.

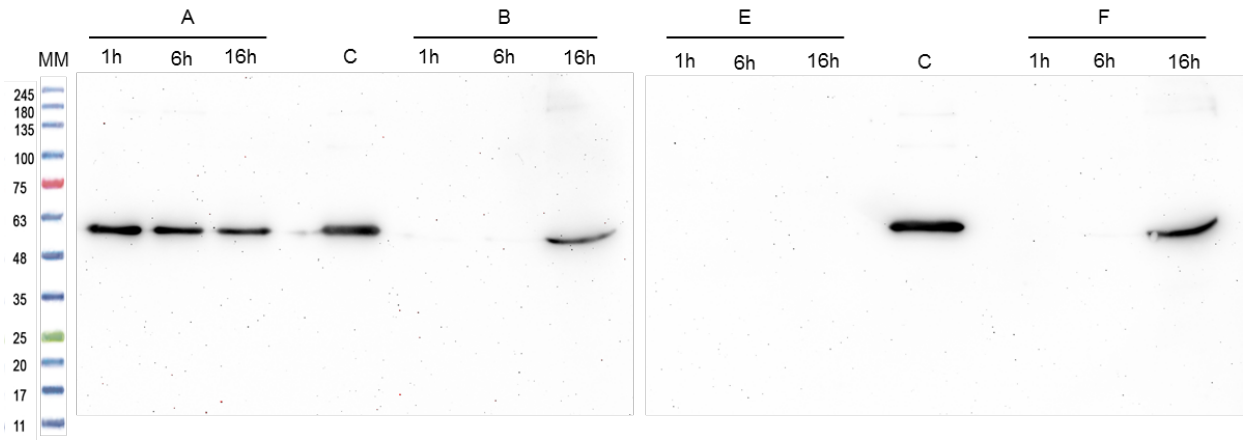


FIGURE IV.14 – Western Blot analysis of 6xHis hPAHwt in different cellular fractions of HepG2 after cell incubation with NP-CS-hPAHwt for 1 h, 6 h and 16h. (A) Culture medium supernatant; (B) whole cell lysate; (E) lysate supernatant and; (F) lysate pellet. The 6xHis hPAHwt was used as control (C). The anti- 6xHis was used as the primary antibody

The obtained data indicate that the 6xHis hPAHwt protein is present in the culture medium supernatant till 16 h incubation, at 37ºC, demonstrating that the protein is maintained (and not degraded) during this time period (Figure IV.14.A). However, only after 16 h of contact it was possible to recover the protein in the whole cell lysate (Figure IV.14.B) and in the lysate pellet (Figure IV.14.F), but never in the supernatant of the cell lysate (Figure IV.14.E). These data indicate that the free hPAHwt and/or the encapsulated form is associated to the cellular membrane. However, the question whether the CS-NPs are outside or inside cellular membrane remains to be clarified.

The absence of PAH in the lysate supernatant observed in this assay (Figure IV.14.E) can be justified by different factors, such as, the need for optimizing the cell culture conditions, more specifically, temperature and/or time of incubation. Another aspect that must be taken into account is the internalization pathway used by the CS-NP. NPs are known to be internalized by the cells through endocytosis during long periods of incubation and, thus latter on they are found in organelles associated to membranes and, only occasionally, free in cytoplasm (170). To clarify the hypothesis of whether the protein, although associated to cell membranes, would be inside or outside the cell, immunocytochemistry assays were performed. However, and as shown in Figure IV.15, the results were inconclusive. We were able to confirm that after 16 h of incubation, the CS-NP-hPAHwt were indeed associated to cell membrane, seen by the green fluorescent dots along the cell membrane marked in red (Figure IV.15.B), but their exact location is still unknown.

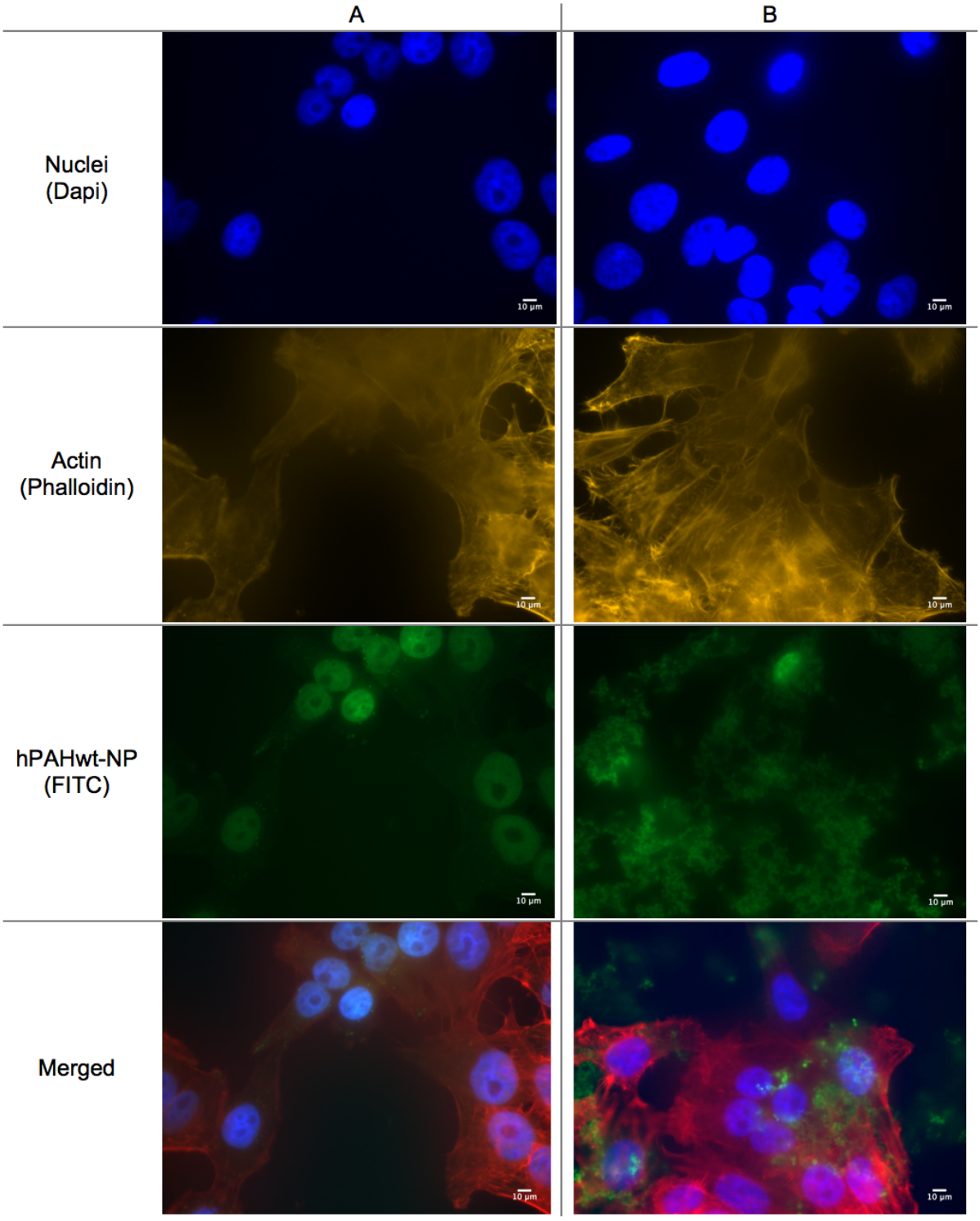


FIGURE IV.15 – Immunocytochemistry assays in HepG2 cells cultured for 16 h, at 37ºC, in the absence (A) and presence of hPAHwt loaded CS-N P (B). Immunofluorescence microscopy was performed by using anti-mouse FITC-conjugated secondary antibody (green) for hPAH. Nuclei and actin ere counterstained with Dapi (blue) and Phalloidin (red).

3.4. STABILITY OF hPAH ACTIVITY

The previous assays showed that the 6xHis hPAH loaded in CS-NP is stable until 16 h of incubation, at 37ºC, when in contact with HepG2 cells. However, the functionality of this protein was still unknown. To evaluate if the biological function of hPAHwt was preserved in the CS-NP formulation the hPAH activity was determined, at different incubation times, in both the cell culture and in total cell lysate. Appropriate control assays (naked hPAHwt in the same assay conditions) were also performed.

To date, measurement of hPAH activity is routinely performed in our lab, using an end point assay and a standard reaction mixture containing the enzyme, Fe^{2+}, L-Phe and BH_4. The L-Tyr production is further determined by HPLC with a time-consuming chromatographic separation of substrate and product. The implementation in our laboratories of the fluorescence-based multi-well assay, developed by Gersting et al (105), has given rise to the possibility of evaluating hPAH activity more rapidly, but as precisely and accurately as the standard methods. This technique uses a fluorescence detection device with an integrated injection system. The differences in the fluorescence properties of the aromatic amino acids L-Phe and L-Tyr, such as emission and excitation wavelengths as well as the quantum yield, allow spectral separation of these substances even in a mixed solution. To confirm spectral separation of the two substances, a calibration curve was determined through detection of fluorescence signal intensities of varying L-Tyr concentrations in the presence of 1 mM L-Phe (Figure IV.16) and as function of increasing L-Phe concentrations (results not shown). The curve showed linear correlation, so, detection of L-Tyr was unaffected by the various L-Phe concentrations.

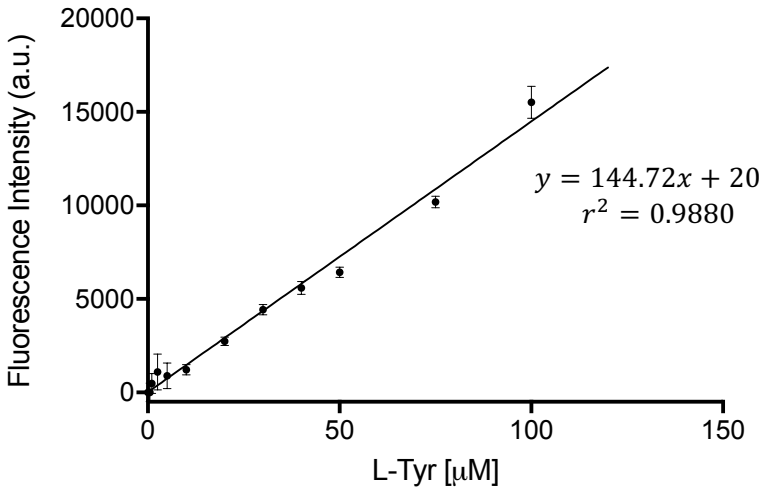


Figure IV.16 – Fluorescence intensity of L-Tyr solutions (0 – 150 μM) in the presence of 1 mM L-Phe. Values are given as the mean ± SD of three independent measurements.

Using the above fluorescence-based multi-well assay the hPAH activity was determined after 1, 2, 4 and 21h incubation, at 37 ºC, in different mediums namely, SEC buffer, culture medium and cell homogenate. As control the naked hPAHwt was also studied in the same conditions. The obtained activities were compared with the values obtained for the hPAHwt maintained at 4ºC, which were considered 100%. The obtained data are presented in Figure IV.17.

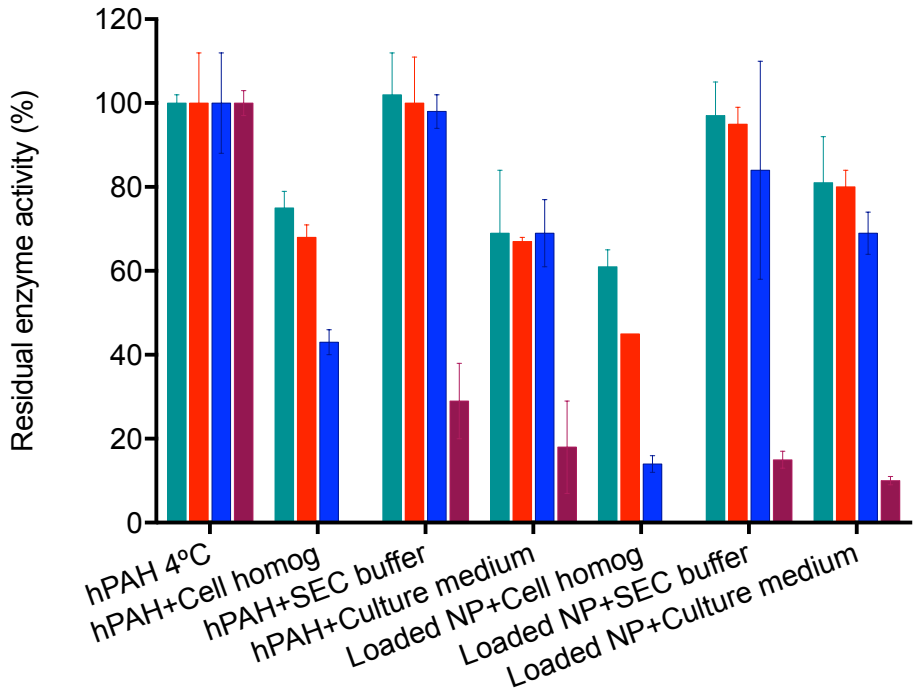


Figure IV.17 – Residual enzyme activity determined after 1 (■), 2 (■), 4 (■) and 21 h (■) incubation of naked hPAHwt and hPAHwt loaded CS-NP, at 37ºC, in different mediums. The enzyme activity of hPAHwt maintained at 4ºC was considered 100%. The data represent the mean and SD of two independent assays.

After 21 h incubation, in all the conditions tested the residual enzyme activity was below 29%. For the other tested conditions no significant differences were observed among the naked and encapsulated hPAHwt, except when the assay was performed in culture medium. In this condition, and when compared with the naked hPAH, the encapsulated hPAHwt seemed to be protected resulting in a higher activity for the 1, 2 and 4 h incubation tested.

CHAPTER V • **CONCLUSION AND FUTURE PERSPECTIVES**

The search for an effective treatment for the severe forms of phenylketonuria, a conformational disorder with loss-of-function, led us to explore distinct approaches that *per se* or in combination could treat the most severe clinical phenotypes that currently do not have an alternative treatment other than the life-long burdensome restrictive diet which has been associated with and long term decline in executive function.

This work has contributed to: (i) hint a new class of pharmacological chaperones acting on the aggregation of the severe p.G46S variant; (ii) optimize experimental conditions for further studies aiming the elucidation of the proteostasis network pathways involved in the degradation of variant hPAH proteins that ultimately can lead to the identification of proteostasis regulators as a new class of small molecules for the treatment of PKU and; finally (iii) confirm that loaded CS nanoparticles, previously developed by the research group, are a potential drug delivery system (DDS) for further studies aiming a novel PKU treatment approach by enzyme replacement therapy.

The *in vitro* model of the aggregation-prone p.G46S variant, allowed the identification of small molecular weight compounds inhibiting p.G46S aggregation (C6 e C18). These compounds belong to an in-house rationally-designed compound library of 22 3HQ derivatives and were designed (and synthesized) by the Bioorganic Group (coordinated by Prof. Pedro Góis; iMed.ULisboa) to specifically interact with the hPAH protein by incorporating in their structure the substrate L-Phe (to confer specificity) and the pharmacophore 3-hydroxyquinolin-2(1H)-one (3HQ) (by its ability to complex iron). The 3HQ derivatives C6 and C18 not only inhibited p.G46S *in vitro* aggregation but also *in cellulo* and are promising compounds to be used scaffolds for further structure refinement. However, the mechanism of binding is still unknown but taking into consideration of previous data obtained by our research group showing that compounds that inhibit the p.G46S aggregation also inhibit the aggregation of the hPAH N-terminal regulatory domain (171) we hypothesize that C6 and C18 will probably have a similar mechanism of binding to the hPAH regulatory domain. To confirm our hypothesis these molecules will be soon sent to Professor Wyatt Yue (University of Oxford), under a collaborative project, to perform co-crystallization assays (C6 and C18 and the N-terminal regulatory domain). Moreover, the identification of these modulators could lead to a new class of pharmacological chaperones that could be further used to study synergisms between the use of proteostasis regulators and pharmacological chaperones.

In conformational disorders with loss-of-function the normal balance between folding and degradation machineries (proteostasis) is displaced towards the accelerated degradation of the misfolded protein due to their decreased stability and high tendency to aggregate. Therefore, small molecules modulating, not only the conformation (pharmacological chaperones), but also the interactions of misfolded proteins with molecular chaperones (proteostasis regulators), will enhance the concentration and/or location of the target proteins

thus contributing to alleviate disease pathogenesis. As such this project aimed to study in HEK293T cells (as they not express hPAH constitutively), the p.G46S interactions with proteins of the folding and the cell protein quality control system as putative proteostasis therapeutic targets. Different interaction patterns, between p.G46S and the tested molecular chaperones (Hsp90AB1, HspA8, HspA2, HspH1 and SQSTM1), were observed. Although no conclusive results came up, these experiments allowed the optimization of the experimental conditions, which might open doors to further studies using the modulation of proteostasis regulators aiming the cellular rescue of variant hPAH proteins.

Finally, the previously developed nano-DDS (CS-NP loaded with hPAHwt) was characterized and evaluated. At a first evaluation the CS-NPs did not seem to be uptaked by cells. Although, the immunocytochemistry assay showed that they might be associated to the cell membrane, it is still unclear if they are associated to internal cell membrane structures. As such, confocal microscopy assays should be performed in order to clearly assess the cellular localization of the developed hPAHwt loaded CS-NPs. Interestingly, our preliminary assays, regarding a protective effect of CS-NPs, showed that in fact the nanoencapsulated hPAHwt presented a higher activity (after 4 h incubation in culture medium, at 37°C) than the naked protein (in the same experimental conditions). In terms of desirable effects of the DDS, the protective effect is probably more important when compared to NPs cell internalization, once our main goal is to have a L-Phe degradation capacity (hPAH enzymatic activity) but not necessarily occurring inside the cell (hepatocyte). Although this experimental approach showed good perspectives, in order to achieve an accurate point of star, a higher number of assays (n) are needed.

In conclusion, this work allowed the identification of small molecular weight compounds modulators for severe forms of PKU, the optimization of a model to identify proteostasis regulators involved in loss-of-function disorders, as well as, a vision of a possible synergic approach with the use of proteostasis regulators and pharmacological chaperones and, finally, optimized techniques to continue developing strategies to move forward with the enzyme replacement therapy approach.

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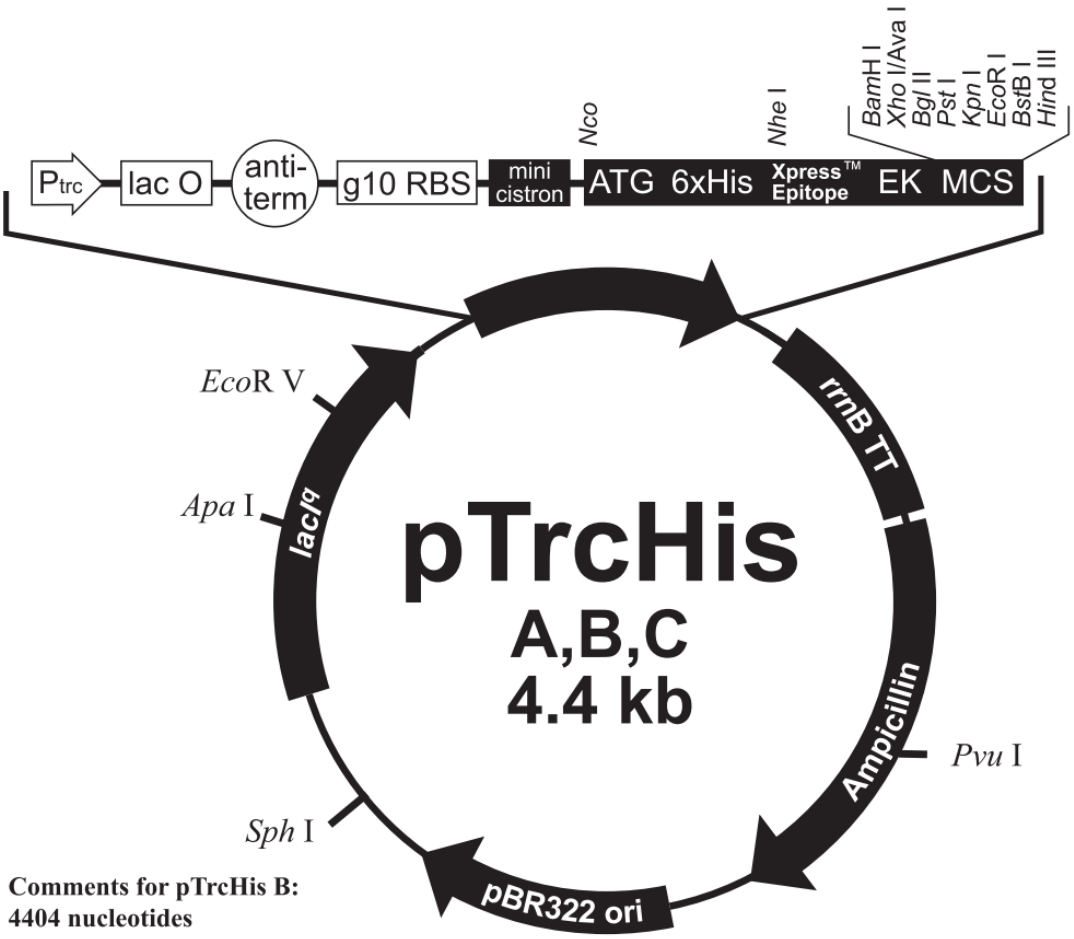
APPENDIX

1. Vector used for expression of hPAHwt



Comments for pTrcHis B:
4404 nucleotides

trc promoter: bases 191-221
lac operator: bases 228-248
*rrn*B anti-termination sequences: bases 264-333
T7 gene 10 translational enhancer: bases 346-354
Ribosome binding site: bases 370-374
Mini-cistron: bases 383-409
Polyhistidine and enterokinase cleavage site: bases 425-504
Xpress™ epitope: bases 482-505
Multiple cloning site: bases 515-554
*rrn*B transcriptional termination sequence: bases 637-794
Ampicillin resistance ORF: bases 1074-1934
pBR322 origin: bases 2079-2752
lac I^q ORF: bases 3406-4365

Figure III.1 – Map and features of pTrcHis vector (Invitrogen) (172).

2. Vector used for the expression of hPAH-G46S fusion protein

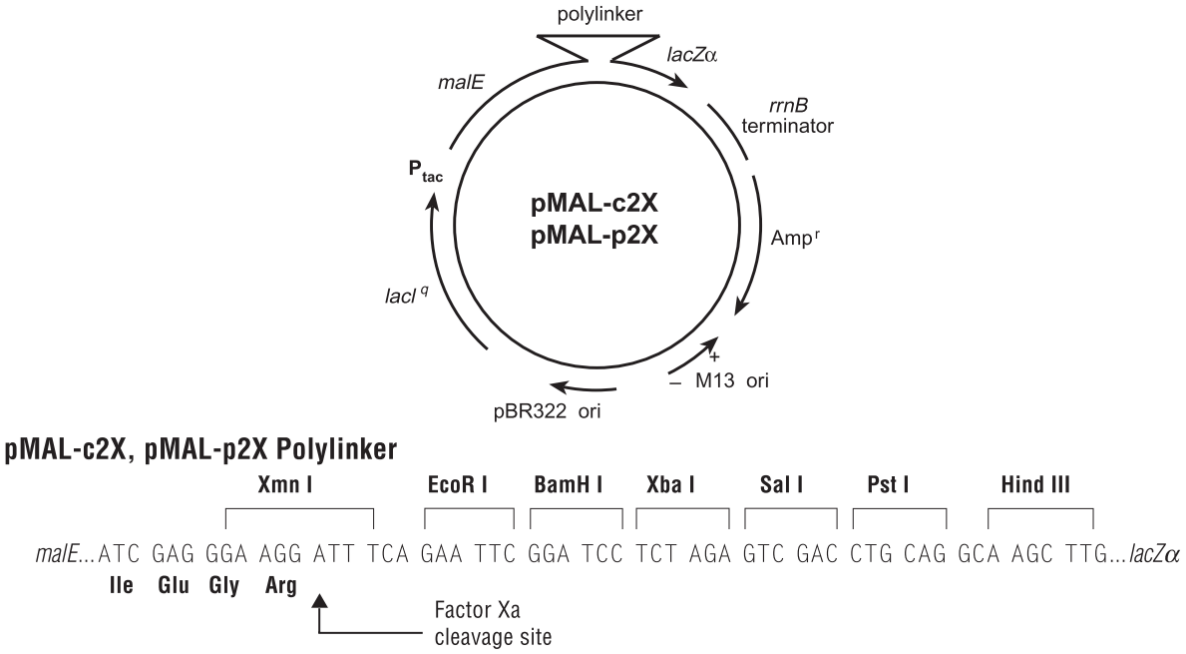
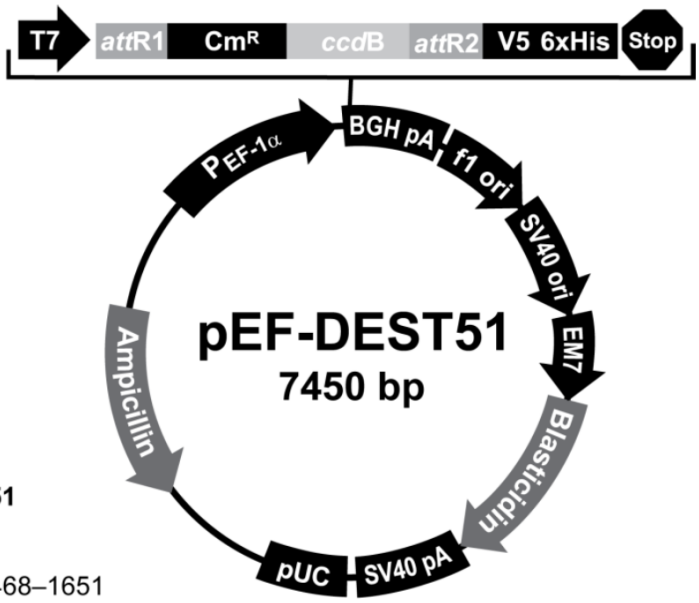


Figure III.2 – Map and features of pMAL-c2X (England BioLabs). Unique restriction sites are indicated (173).

3. Vector used for the expression of molecular chaperone in eukariotic systems.



Features of pEF-DEST51
7450 nucleotides

EF-1α promoter: bases 468–1651
T7 promoter: bases 1668–1687
*att*R1 recombination site: bases 1718–1842
Chloramphenicol resistance gene: bases 1951–2610
*ccd*B gene: bases 2952–3257
*att*R2 recombination site: bases 3298–3422
V5 epitope: bases 3448–3489
6xHis tag: bases 3499–3516
BGH polyadenylation region: bases 3542–3769
f1 origin: bases 3815–4243
SV40 early promoter and origin: bases 4248–4617
EM7 promoter: bases 4626–4681
Blasticidin resistance gene: bases 4700–5098
SV40 early polyadenylation region: bases 5253–5386
pUC origin: bases 5769–6431
Ampicillin resistance gene (*bla*): bases 6576–7436 (c)
bla promoter: bases 7537–71 (c)
(c) = complementary strand

Figure III.3 – Map and features of pEF-DEST51 Gateway™ Vector (156).

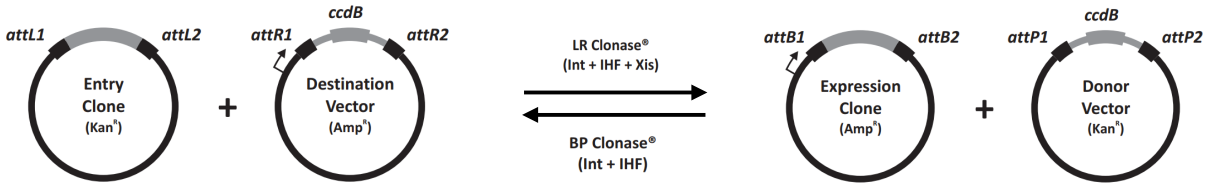


Figure III.4 – The Gateway™ Recombination system (157).